UNIVERSITY OF EDINBURGH

PHYSICOCHEMICAL STUDIES ON POLYSACCHARIDES

by

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FOREWORD

This thesis consists of reprints of published work and the manuscript of a paper which has been accepted for publication, together with a brief outline of the general field of investigation. The experimental work has been carried out mainly by research students or post-doctoral research workers under the writer's direct supervision. In the majority of papers, however, the writer has contributed in some part to either the actual experimental work or to the calculations.

PUBLICATIONS.

1. "The Size and Shape of Some Polysaccharide Molecules", by C.T. Greenwood, Advances in Carbohydrate Chemistry, 1952, 7, pp. 289-332.

2. "A Simple Electrometer for Small-scale Potentiometric Titrations", by D.M.W. Anderson and C.T. Greenwood, Chemistry and Industry, 1953, p. 476.

3. "The Characterization of Branched α-1:4-Glucosans", by D.M.W. Anderson and C.T. Greenwood, Chemistry and Industry, 1953, p. 642.

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5. "Physicochemical Studies on Starches. Part I. The Characterization of the Starch present in the Seeds of the Rubber Tree, Hevea brasiliensis", by C.T. Greenwood and J.S.M. Robertson, Journal of the Chemical Society, 1954, pp. 3769-3778.

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15. "Mechanism of the Degradation of Potato Amylose by β-Amylase", by J.M.G. Cowie, I.D. Fleming, C.T. Greenwood and D.J. Manners, Chemistry and Industry, 1957, pp. 634-635.

16. "The Degradation of High Polymers", by W.A.J. Bryce and C.T. Greenwood, Journal of Polymer Science, 1957, 25, pp. 480-483.

17. "Physicochemical Studies on Starches. Part V. The Effect of Acid on Potato Starch Granules", by J.M.G. Cowie and C.T. Greenwood, Journal of the Chemical Society, 1957, pp. 2658-2665.

18. "Physicochemical Studies on Starches. Part VI. Aqueous Leaching and the Fractionation of Potato Starch", by J.M.G. Cowie and C.T. Greenwood, Journal of the Chemical Society, 1957, pp. 2862-2866.

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22. "Physicochemical Studies on Starches. Part X. The Molecular Weight of the Water-soluble Polysaccharides of Sweet Corn, Zea mays", by C.T. Greenwood and P.C. Das Gupta, Journal of the Chemical Society, 1958, pp. 703-707.

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24. "Physicochemical Studies on Starches, Part XII. The Molecular Weight of Glycogens in Aqueous Solution", by W.A.J. Bryce, C.T. Greenwood, I.G. Jones and D.J. Manners, Journal of the Chemical Society, 1958, pp. 711-715.

25. "Physicochemical Studies on Starches. Part XIII. The Fractionation of Oat and Wheat Starches", by A.W. Arbuckle and C.T. Greenwood, Journal of the Chemical Society, 1958, pp. 2626-2629.

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27. "Physicochemical Studies on Starches. Part XV. The Action of β-Amylase on Glycogen as shown by Molecular-weight Distribution", by W.A.J. Bryce, J.M.G. Cowie, C.T. Greenwood and I.G. Jones, Journal of the Chemical Society, 1958, pp. 3558-3563.

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29. "Physicochemical Studies on Starches. Part XVII. The Effect of Oxygen-treatment on the β-Amylolysis of Amylose", by W. Banks, C.T. Greenwood and J. Thomson, Chemistry and Industry, 1959, p. 928.

30. "Physicochemical Studies on Starches. Part XVIII. The Properties of Amylose as Related to the Fractionation and Subfractionation of Starch", by W. Banks, C.T. Greenwood and J. Thomson, Die Makromolekule Chemie, 1959, 31, pp. 197-213.

31. "A Comparison of the Starches from Barley and Malted Barley", by C.T. Greenwood and J. Thomson, Journal of the Institute of Brewing, 1959, pp. 346-353.

32. "Physicochemical Studies on Starches. Part XIX. The Fine-structure of the Amylose Component of Starch", by W. Banks and C.T. Greenwood, Die Stärke, 1959, 11, pp. 294-298.

33. "The Starch of the Tuber and Shoots of the Sprouting Potato", by W. Banks and C.T. Greenwood, Biochemical Journal, 1959, 73, pp. 237-241.

34. "Physicochemical Studies on Starches. Part XX. On the Existence of an Anomalous Amylopectin in Starch", by W. Banks and C.T. Greenwood, Journal of the Chemical Society, 1959, pp. 3436-3439.

35. "Physicochemical Studies on Starches. Part XXI. Observations on Z-Enzyme", by W. Banks, C.T. Greenwood and I.G. Jones, Journal of the Chemical Society, 1960, pp. 150-155.

36. "The Molecular Properties of the Components of Starches", by C.T. Greenwood, Die Stärke, 1960, in the press.

PHYSICOCHEMICAL STUDIES ON POLYSACCHARIDES.

The work described in this thesis deals with aspects of the physical chemistry of polysaccharides, with special reference to the determination of their molecular size. The general problems involved in such studies have already been summarized, and the pertinent literature reviewed (1, 12b).* Whilst most of the work detailed here has been carried out on starch and its components, some other polysaccharides have been investigated. (No attempt, however, has been made to investigate cellulose or its derivatives.)

The Determination of Molecular Size.- Methods for determining the molecular size and shape of polysaccharides have been outlined (1, 12a, 12b).

In this work, number-average molecular weights have been obtained from isothermal distillation experiments (11) for materials with a molecular weight of less than 20,000, and from osmotic pressure measurements for other samples. Procedures for the accurate determination of small osmotic

* The numbers in this section refer to the papers listed previously.

pressures have been described (5, 17), and errors inherent in this type of measurement critically discussed (6).

Weight-average molecular weights have been obtained from lightscattering and from sedimentation and diffusion measurements. A method for measuring the light scattered from large polysaccharides in aqueous solution has been perfected (22, 28). Ultracentrifugal measurements have been carried out on a wide variety of polysaccharides to determine their sedimentation coefficients. Sedimentation velocity diagrams have also been analysed to give molecular weight distributions, and the procedure entailed in these calculations systematized (27). A new micro-cell procedure for measuring diffusion coefficients has been described (28).

Viscosity measurements have been extensively used to characterize molecular size, particularly for the starch components; a detailed procedure for both the unsubstituted components and their derivatives has been given (5, 17).

Studies on Starch.

Problems outstanding in the field of the physical chemistry of starch and also most of the important literature in the field have been reviewed (12a). The work carried out on starch by the author's group has been in an attempt to answer some of the problems raised in the above Review.

The characterization of starch and its components.- For fundamental studies, an accurate method of determining the purity of the starch components is essential. This is best accomplished by potentiometric measurements of the iodine-binding power of these polysaccharides. For this purpose, a simple, but sensitive, electrometer was developed for differential potentiometric titrations (2). This apparatus enabled the extremely low iodine-binding power of branched α-1:4-glucosans to be adequately characterized for the first time (3). Later developments resulted in a valve microvoltmeter, and results of a high degree of accuracy were obtained for a wide variety of starches and various other α-1:4-branched glucosans (8). The iodine-binding power of amylose is also very satisfactorily measured in this manner (5, 17, 18, 20, 21, 30). (Recently, the nature of the solid amylose-iodine complex has been investigated by measurements of its infrared absorption spectrum (19). A comparison of the spectra of various halogen-amylose complexes suggested that halogen-oxygen interaction was occurring. This result implied that the most probable conformation of the glucopyranose ring in amylose is the chair-form in which only one substituent is axial to the plane of the ring.)

One of the characteristics of the branched amylopectin component is its average length of unit-chain. It was shown (7) that this may be rapidly and accurately estimated by oxidation with potassium metaperiodate when the experimental conditions are closely defined. A wide variety of starches were analysed by this method.

Viscosity measurements have been used extensively as a rapid, but accurate, method for characterizing the molecular size of amyloses. Limiting viscosity numbers $[\eta]$ measured in 1M potassium hydroxide were converted to degrees of polymerization ($\overline{DP}$) by the equation $\overline{DP} = 7.4 \times [\eta]$. This latter relation was obtained by comparing the limiting viscosity number for a series of samples of amylose with the molecular weight determined from osmotic pressure measurements on the corresponding acetate (18). Although fractionated samples were not used, this relation appears to be as reliable as any other in the literature (cf. 12a).

An original method of characterizing both amylose and amylopectin by measurements of their sedimentation coefficients in 0.2M potassium hydroxide was proposed (14). This method is very convenient for comparing the molecular size of amylopectins (23, 31, 33).

Weight-average molecular weights for amylose and

amylopectin in aqueous solution have been obtained from lightscattering measurements (36). These measurements also allow estimates to be made of the radii of gyration of the molecules.

Sedimentation coefficients of amylopectin may also be measured in water, but in the case of potato amylopectin, ionic effects due to the phosphate groups present can cause complications (33). This effect has been studied in detail by viscosity, lightscattering and sedimentation measurements (36).

Isolation of starches.- It is essential to isolate and purify starches by methods which avoid any inadvertent degradation or modification of the components. This is particularly true for amylose (33). A physical method has been evolved, however, to remove the contaminating protein associated with a wide variety of starches. This procedure (5, 9), which involves denaturating protein at a toluene- or butan-1-ol-water interface, has been found to be applicable to any granular starch.

The fractionation of starch.- Early work (5) showed that fractionation was satisfactory when starch was dispersed into solution, the amylose precipitated as the thymol complex, and then recrystallized as the butan-1-ol complex.

Pure amylopectin was obtained from the supernatant liquor. A critical study of the fractionation of potato starch was later carried out (18). The efficiency of aqueous leaching of the granules at various temperatures was followed by measuring the purity and molecular weights of the resultant components. These studies showed conclusively that aqueous leaching was both inefficient and incomplete compared to the method involving dispersion of the granular structure and the formation of a thymol-amylose complex. Further, the aqueous-leached amyloses were of smaller molecular size than those obtained from dispersion. The latter method gave potato amylose with an average degree of polymerization of about 4,000 anhydroglucose units; a value much higher than others in the literature (cf. 12a). The reasons for this were investigated (20). Degradation of amylose was found to occur readily when oxygen or air was present during a fractionation; an inert atmosphere was essential. The amylose from commercial potato starch was also shown to be much smaller than that from the laboratory-prepared samples.

Sub-fractionation of potato starch (20) indicated the presence of an anomalous amylopectin in the supernatant liquor from the recrystallization of the initial amylose-complex. This material was subsequently examined in more

detail (34), and shown to have a significantly lower average chain-length and β-amylolysis limit than amylopectin. The polysaccharide, which represented 5 - 10% of the granule, was thought to be inherent in granular structure and to arise from enzymic modification of the amylopectin.

Although potato starches readily swell and disperse into solution, cereal starches require some form of pretreatment before this occurs. M-potassium hydroxide at 0° was used for this purpose in earlier studies (23, 25), but more recent work has shown that treatment with liquid ammonia is better (30). This method, which does not cause any degradation of the amylose, has been applied very successfully to a wide variety of starches (30, 31, 36), and would appear to be an essential prerequisite to the fractionation of any starch.

Granular structure.- A fundamental problem is the nature of the starch granule and the method of association of the component amylose and amylopectin. One approach is to study the effect of acid-treatment of granules on the properties and molecular size of the components. Two varieties of potato starch were investigated in this manner (17). Both behaved similarly; under the conditions used, the granular structure was apparently unchanged, but on fractionation, degradation of both amylose and amylopectin

had occurred. A study of the rates of hydrolysis (theoretical aspects of polymer degradation were considered (16)) showed that preferential degradation of the amylopectin occurred, thus indicating that the outside of the granule was essentially amylopectin. Similar results were obtained when wheat-starch granules were subjected to acid-treatment, but the slower rate of degradation of both components indicated a more compact structure for these granules (26). Evidence from aqueous leaching experiments (18) also indicated the amylopectin-character of the outer portion of the granule.

The fine-structure of amylose.- A survey has been made of the development of ideas on the fine-structure of amylose (32). Amylose obtained by precipitation from a starch dispersion is incompletely hydrolysed to maltose by β-amylase. β-Amylolysis experiments on the amylose fractions obtained by successive aqueous leaching of potato starch showed, however, that at low temperatures the extracted material was completely hydrolysed, whilst with increase in temperature the resultant amylose was larger in molecular size and had a lower β-amylolysis limit (20). It was thus shown conclusively for the first time that amylose was heterogeneous. Similar results were obtained on leaching oat and wheat starches (25), and a randomly-situated barrier

in the larger amyloses was suggested. A more detailed study of this problem (30) showed that when any granular starch has been treated with boiling 80% aqueous methanol, leaching gave a subfraction of amylose, which was completely hydrolysed by β-amylase. The properties of subfractions of amylose from a wide variety of starches have been described (30, 31, 36).

The nature of the barrier in amylose to the action of β-amylase has been in dispute (12a), but it can be effectively removed by Z-enzyme. However, barriers could be introduced by passing molecular oxygen through a heated aqueous solution of linear amylose (29). Further, fractionation in the presence of air introduced barriers (29), and pretreatment procedures could also cause them (30). The possibility existed therefore that the barriers were artefacts, although natural barriers (e.g. phosphate (33)) could not be excluded. However, all barriers could be effectively removed by Z-enzyme (32). Z-enzyme was therefore α-amylolytic in action, or was specific for oxidised anhydroglucose units.

The action-pattern of Z-enzyme was studied (35). It was found from viscosity measurements that the enzyme degraded, in a manner consistent with a random hydrolytic action, both linear amylose and amylose containing a barrier

to β-amylase. Lightscattering measurements showed that limited hydrolysis of amylopectin β-limit dextrin also occurred, whilst there was no action on the β-limit dextrin of glycogen. It was therefore shown conclusively for the first time that the hydrolytic action of Z-enzyme on amylose and amylopectin was indistinguishable from that of an α-amylase.

The nature of the barrier in amylose remained unknown as Z-enzyme was nonspecific in character, but a combination of a natural and artificial barrier was thought to be most likely (32, 35).

In an attempt to establish whether the natural barrier in potato amylose was due to branching, the hydrodynamic behaviour was studied of a series of amylose fractions of differing molecular weight and β-amylolysis limit (36).

Properties of starches.- Although potato starch has been extensively investigated, 14 other starches from a wide variety of botanical sources have been examined (5, 23, 25, 36). Further, a comparison of the starches from barley and malted-barley (31) has given the first full insight into the enzymatic changes occurring in starch during malting. Changes occurring in the fine-structure of the components of starch during growth of the potato have also been investigated in detail for the first time(33).

Action-pattern of β-amylase.- Physical methods provide the only satisfactory means for determining the detailed action-pattern of an enzyme. A preliminary attempt in this direction was made by studying the action of β-amylase on amylose (21). Sedimentation measurements indicated that hydrolysis occurred by an essentially single-chain mechanism. These studies suffered, however, from the fact that only mean sedimentation coefficients were measured (cf. 35).

More recently, when methods were available for analysing sedimentation patterns in terms of molecular weight distribution, the action-pattern of β-amylase on glycogen was investigated on a molecular basis for the first time (27). The molecular weight distribution for the original sample was compared with that for two dextrins. The curve for the resultant β-limit dextrin showed that all glycogen molecules in the sample, independently of molecular size, were hydrolysed to the same relative extent. Examination of an intermediate dextrin showed that also during β-amylolysis all the polysaccharide molecules were degraded to the same extent.

Other α-a:4-branched glucosans.- Detailed studies have been made of other starch-type polysaccharides. The water-soluble polysaccharide of sweet corn, Zea mays, has been shown to be essentially homogeneous, with a structure more similar to that of a glycogen than an amylopectin (22).

Glycogen has also been studied in detail after its ideal hydrodynamic behaviour had been shown earlier (14). When a large number of samples were examined (24), the presence of more than one component in some samples was observed for the first time. This resulted in large differences between molecular weights from sedimentation-diffusion and lightscattering measurements. This latter problem was critically studied by evaluating apparent molecular weight distributions of various samples of rabbit liver glycogen (28). In addition, it was shown conclusively that glycogen extracted by cold trichloroacetic acid is more representative of native glycogen than that obtained by hot alkali.

Studies on Polysaccharides other than Starch and Cellulose.

An examination has been made of the capsular polysaccharide from an amylolytic sheep rumen streptococcus (4). The material was homogeneous on ultracentrifugation, and a molecular weight of 90,000 was calculated from sedimentation and viscosity measurements.

A preliminary account has been given (10) of a new phenomenon observed during sedimentation studies on a plant gum of the species Khaya grandifolia. Samples of this gum showed molecular aggregation effects, due to the presence of heavy metal ions.

The complex structure of the seaweed polysaccharide, laminarin, has been shown when the results of measurements of the molecular weight of methylated samples by the isothermal distillation method were compared with those of chemical end-group assay.

A wide range of methylated hemicellulose derivatives has also been studied by the isothermal distillation method. (Most of these materials possess molecular weights in the range of 2,000 - 20,000, and therefore this method is one of the few available.) The results of these determinations have been included in publications of Dr. G.O. Aspinall and his collaborators as shown:

Polysaccharide	No. units non-reducing end-group	$\overline{DP}$ by isothermal distillation	Ref.
Wheatstraw xylan	40-45	50±3	J., 1954, 1731
Hemicellulose A (Beechwood)	70	70±4	J., 1954, 1734
Barley glucosan	> 200	100±5	J., 1954, 3519
Norway spruce xylan	ca. 100	100±5	J., 1956, 3744
Wheatstraw hemicellulose	50	74±4	J., 1956, 3830
Barley husk hemicellulose	-	66±4	J., 1957, 4188
Glucomannan (Sitka spruce)	35	49±3	J., 1957, 4444
Jute hemicellulose I	40	123±6	J., 1958, 3627

THE SIZE AND SHAPE OF SOME POLYSACCHARIDE MOLECULES

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Contents

I. Introduction

The polysaccharides form a group of substances widely distribu throughout Nature, and they provide an extensive and ever-increa field of investigation. They vary considerably in the complexity of t structure, and in molecular size and shape. Although early invest tions based on chemical reactions did not indicate the true polym nature of these molecules, physical methods of analysis have now sh that they come into the class of high polymers.

The general problems which arise in the investigation of these s stances include the determination of their structure and their size w in the native state. Organic and biochemical studies, which indicate nature and configuration of the structural units in the polysacchari are a necessary and essential preliminary to the physico-chemical de minations of molecular size and shape. Each polymer introduces spe problems, but all entail some method of isolation and, therefore, it is necessary to follow any changes occurring in this process or in the for tion of derivatives.

Most work concerning these problems has been carried out on st and cellulose, and certain aspects of the determination of their molec shape and size have already been reviewed.[1] The work carried out other polysaccharides up to the end of 1951 will be covered here.

II. The Determination of Molecular Weight

The range of the molecular weights of the polysaccharides is v great and almost all the conventional methods of determination h been applied. The more usual of these methods and their limit application are summarized in Table I. As reviews of the methods[2] the techniques[2a] involved are available it will not be necessary to des these in detail, but a short outline will be given below.

Chemical methods of end-group assay give the lower limit of molecular weight, and when combined with physical determinat may give the degree of branching of the polymer. If the molecu unbranched, chemical methods give the true molecular weight, but fact that the accuracy of the method decreases in direct proportion t

(1) R. W. Kerr, "The Chemistry and Industry of Starch," 2nd edition, Aca Press, N. Y. (1950); P. O. Kinell and B. G. Rånby, *Advances in Colloid Scie* 161 (1950).

(2) P. Johnson, *Ann. Repts. Progress Chem. (Chem. Soc., London)*, **43**, 30 (P. M. Doty and H. Mark, *Ind. Eng. Chem.*, **38**, 682 (1946); A. E. Alexander a Johnson, "Colloid Science," Oxford Univ. Press (1949).

(2a) A. Weissberger, "Physical Methods of Organic Chemistry," volu Interscience Publishers, New York (1949).

TABLE I
Methods of Molecular Weight Determination

Method	*Molecular weight range most suited for method*	*Molecular weight average*
1. Chemical methods	Molecules containing more than one end-group per one hundred units.	Number-average
2. Cryoscopic and ebullioscopic methods	Molec. wt. less than 20,000	Number-average
3. Vapor pressure lowering	Molec. wt. less than 20,000	Number-average
4. Osmotic pressure	20,000–500,000	Number-average
5. Viscosity	Any range	Weight-average or Viscosity-average
6. Light-scattering	Molec. wt. greater than about 50,000	Weight-average
7. Sedimentation velocity and diffusion	Any range	Weight-average or Z-average
8. Sedimentation equilibrium	Mol. wt. greater than 10,000	Weight-average or Z-average

size of the molecule usually limits its application in this way to relatively small molecules. Sources of error in the method of end-group assay are primarily the lack of complete reaction of all end-groups, side reactions including degradation of the molecule, and sometimes an insufficient knowledge of the nature of the end-groups.

In the case of polysaccharides, where a potential aldehyde group exists at an end of the molecule, reagents reacting specifically with this group have been widely used. Such reagents include iodine, copper-reducing agents and substances capable of forming colored complexes.[3] In addition, methods have been developed for estimating the non-reducing end-groups. The most frequently used is that originated by Haworth and Machemer[4] in which the polysaccharide is methylated, hydrolyzed by acid, and the resultant methylated monosaccharides converted to methyl glycosides, separated by fractional distillation and estimated. The ratio of the different components gives an idea of the molecular structure and the average chain length. Recent important modifications[5] of this method using chromatography to estimate the simple

(3) For a critical survey of these methods see: Sylvia Lansky, M. Kooi and T. J. Schoch, *J. Am. Chem. Soc.*, **71**, 4066 (1949).

(4) W. N. Haworth and H. Machemer, *J. Chem. Soc.*, 2270 (1932).

(5) A. E. Flood, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 1679 (1948); E. L. Hirst and J. K. N. Jones, *ibid.*, 1659 (1949); L. Hough, J. K. N. Jones and W. H. Wadman, *ibid.*, 2511 (1949); E. L. Hirst, L. Hough and J. K. N. Jones, *ibid.*, 928 (1949).

methylated sugars have been described. Alternatively, periodate oxid tion[6] of the free polysaccharide, which liberates formic acid in an amou proportional to the number of pyranose-end-groups, may be employe This method has the advantage of not requiring the preliminary form tion of a derivative of the polysaccharide.

Unless the polymer possesses a low molecular weight, the classic methods of boiling-point elevation and freezing-point depression are n suitable because the temperature changes which have to be measured a too small. These methods count the number of molecules in solutio and consequently extraneous molecules of low molecular weight exe exactly the same effect as molecules of high polymer and cause lar errors. Similar objections apply to the methods, such as isotherm distillation, which depend upon the lowering of vapor pressure. Pro ably the upper limit of molecular weight that it is advisable to attempt measure by these methods is of the order of 20,000.

Although osmotic pressure determinations depend upon the sam thermodynamic principles, the disadvantages of the methods outline above do not occur; the pressure concerned is much more easily measure and impurities exert no osmotic effect if they penetrate the membra that is employed. In fact, the osmotic method is probably the mo convenient and reliable of all and the one most capable of being develope to the highest degree of precision.

According to Van't Hoff's law, the osmotic pressure, Π, for an ide dilute solution is given by the expression:

$$\Pi = RT.C/M \quad (1$$

Polymer solutions, even at very low concentrations, show considerabl deviations from this law, largely due to their abnormally high entropy dilution. It has been calculated on this basis[7] that the osmotic pressure-concentration equation should be of the continued series form:

$$\Pi = RT.C/M + BC^2 + \cdots \quad (2$$

where B and higher coefficients are constants depending upon the solvent-solute interaction. From this equation, which has considerable exper mental support, molecular weights are calculated using the modifie Van't Hoff law:

$$M = RT/\lim_{c \to 0} (\Pi/C) \quad (3$$

(6) F. Brown, Sonia Dunstan, T. G. Halsall, E. L. Hirst and J. K. N. Jone *Nature*, **156**, 785 (1945); K. H. Meyer and P. Rathgeb, *Helv. Chim. Acta*, **32**, 11 (1949); A. L. Potter and W. Z. Hassid, *J. Am. Chem. Soc.*, **70**, 3488 (1948).

(7) P. J. Flory, *J. Chem. Phys.*, **13**, 453 (1945); M. L. Huggins, *J. Am. Chem. So* **64**, 1712 (1942); T. Alfrey and P. Doty, *J. Chem. Phys.*, **13**, 77 (1945); A. R. Mille *Proc. Camb. Phil. Soc.*, **38**, 109 (1942), **39**, 54, 131 (1943).

The usual procedure adopted is to measure Π, at a given temperature, for several concentrations (below 1 g. per 100 ml. solution), and then to plot Π/C against C, the intercept on the Π/C-axis then giving the $\lim_{c\to 0} (\Pi/C)$. As the osmotic pressure is inversely proportional to the molecular size, errors in measurement become larger as the molecular weight of the solute increases, and in addition, difficulties arise in the extrapolation of the Π/C versus C curve to infinite dilution.[8] The osmotic pressure can be determined using either a dynamic or a static method of measurement, but the latter is probably the more accurate of the two. (A recent modification involves weighing the solvent entering the solution.) The limits of the osmotic method are fixed by the reproducibility of the results and the sensitivity of the measuring device for high molecular weights, and by the preparation of a suitable membrane for low molecular weights. The most favorable range of molecular weights is probably between 20,000 and 500,000, although this is by no means the real limits of the method.

It is possible to avoid the effect of the presence of impurities of low molecular weight by employing methods which are sensitive to the weight of the solute particles, and these methods are considered next.

Viscosity measurements have been widely used as a method for determining molecular weights on account of the ease with which they can be carried out. Although the method is simple it is not absolute and requires calibration. The characteristic quantity used in these measurements is the specific viscosity (η_{sp}) defined by Staudinger[9] as $\eta_{sp} = (\eta - \eta_0)/\eta_0$ where η, η_0 are the viscosities of solution and solvent respectively. For concentrations of solute of less than 1 g. per 100 ml., the variation of specific viscosity with concentration C, can usually be expressed by an equation of the form

$$\eta_{sp} = AC + BC^2 \tag{4}$$

where A and B are constant for a given polymer–solvent system. The value of η_{sp}/C at zero concentration was defined by Kraemer[10] as the intrinsic viscosity, $[\eta]$, thus:

$$[\eta] = \lim_{c\to 0} (\eta_{sp}/C) \tag{5}$$

By measuring η_{sp} at several concentrations, the intrinsic viscosity can be evaluated by extrapolation from the graph of η_{sp}/C versus C. The

(8) For a discussion of this point see G. Gee, *Ann. Repts. Progress Chem.* (*Chem. Soc., London*), **39**, 7 (1942).

(9) H. Staudinger, "Die Hochmolekularen Organischen Verbindungen," Springer, Berlin (1932).

(10) E. O. Kraemer, *Ind. Eng. Chem.*, **30**, 1200 (1938); W. D. Lansing and E. O. Kraemer, *J. Am. Chem. Soc.*, **57**, 1369 (1935).

empirical relationship between the intrinsic viscosity and molecul
weight proposed by Staudinger[9] has been recently modified to the mo
general equation

$$[\eta] = KM^{\alpha} \quad (6$$

where K and α are constant for a given polymer–solvent system.[11] Th
equation reduces to that of Staudinger when $\alpha = 1$. The modifie
equation is supported by much experimental evidence and values
α between 0.5 and 1.5 have been found. It is the most satisfactory rel
tionship between the viscosity and the molecular weight.

Recent theoretical developments have related the turbidity of polym
solutions to the size and shape of the dissolved molecules. The ligh
scattered by a solution in excess of that scattered by pure solvent (τ) ca
be shown[12] to be related to the molecular weight by the continued serie
equation

$$HC/\tau = 1/M + 2BC + \cdots \quad (7$$

where H and B are constants. This dependence of the reciprocal of th
turbidity upon the concentration is analogous to the osmotic equation
and the value of $\lim_{c \to 0} (C/\tau)$ is used to evaluate the molecular weight
Both equations also have the same value for the constant B. In certain
circumstances, provided one dimension of the scattering particle is larger
than about $\frac{1}{20}$ of the wave-length of the incident light, it is possible to
obtain an estimate of the shape of the molecule by a study of the dis-
tribution of the scattered light. Another method of determining the
shape of the dissolved polymer is by observing and evaluating the
depolarization of the scattered light.[13] These methods have already
been applied with success to the study of proteins[14] and synthetic poly-
mers,[15] and may be expected to have a wide application in the field of
polysaccharides. They hold particular promise for following reactions
in solution, for gaining evidence of degradation during the formation of
derivatives, and for detecting association in solution.

Dynamic or equilibrium measurements in the ultracentrifuge can be

(11) H. Mark, *Z. Elektrochem.*, **40**, 449 (1934); "Der feste Korper," Hirzel, Leipzig, 1938; R. Houwink, *J. prakt. Chem.*, **157**, 15 (1940).——Concentrations are now usually expressed in terms of grams per 100 ml. solution, although Staudinger uses base-moles per liter (where one base-mole is the weight of polymer in grams equal to the molecular weight of the repeating unit) and consequently the value of K is different from that of the original Staudinger constant denoted by K_m.

(12) P. Debye, *J. Applied Phys.*, **15**, 338 (1944).

(13) P. Doty and H. S. Kaufmann, *J. Phys. Chem.*, **49**, 583 (1945).

(14) P. Doty and J. T. Edsall, *Advances in Protein Chem.*, **6**, 35 (1951).

(15) P. Outer, C. I. Carr and B. H. Zimm, *J. Chem. Phys.*, **18**, 830 (1950).

used to determine the molecular weight of a polymer, and also to obtain information regarding the polydispersity of the substance and the shape of the molecules in solution.

It was shown by Svedberg[16] that the molecular weight of a homogeneous polymer could be calculated from measurements of the rate of sedimentation and of diffusion by the expression:

$$M = RTS/D(1 - V\rho) \tag{8}$$

where S is the sedimentation constant, D is the diffusion constant, V is the partial specific volume of the solute, and ρ the density of the solution. In the case of filamentous molecules, interaction between molecules usually occurs with the consequent dependence of sedimentation and diffusion constants on concentration. This effect can be eliminated to a great extent by carrying out measurements at low concentration, and extrapolating to infinite dilution.[17] The value of the molecular weight obtained for a polydisperse system can vary, as the measured values of the sedimentation and diffusion constants are characteristic for one species in the mixture, or for some average value (related to the different molecular weight averages). Assuming that the partial specific volume is independent of molecular size, nine different values for the molecular weight can be obtained in this manner, the general formula being:

$$M_{\mu\nu} = \frac{RT}{(1 - V\rho)} \cdot \frac{S_\mu}{D_\nu} \tag{9}$$

where μ, ν denote n-, w-, or Z-averages (see below). These values in turn are related to the shape of the molecules in solution. For a complete discussion of this point, see the papers by Gralén,[17] Singer,[18] Jullander[19] and Kinell.[20]

The different molecular species in a polydisperse polymer solution separate in the ultracentrifuge since molecules of different sizes possess different sedimentation constants. If the variation in S_0 is large the separation enables the amount of each species to be estimated, but if there is small difference in molecular size, diffusion causes an overlapping of the boundaries. The spreading of the sedimenting boundary has been used by Gralén[17,21] to estimate the polydispersity of the material. A measure of this can also be obtained from diffusion measurements.[16]

(16) T. Svedberg and K. O. Pederson, "The Ultracentrifuge," Oxford, 1940.
(17) N. Gralén, Dissertation, Uppsala, 1944.
(18) S. Singer, *J. Polymer Sci.*, **1**, 445 (1946).
(19) I. Jullander, *Arkiv Kemi Mineral. Geol.*, **21A**, No. 8 (1945).
(20) P. O. Kinell, *Svensk Kem. Tid.*, **61**, 19 (1949).
(21) See also P. O. Kinell, *Acta Chem. Scand.*, **1**, 335 (1947).

Sedimentation equilibrium measurements are useful for obtain absolute values for the molecular weight. The lower limit of measu ment is about 10,000, but the ultimate limit for high molecular weig has not yet been reached. When equilibrium between sedimentat and diffusion is established, Svedberg has shown[16] that the molecu weight for a homogeneous polymer can be calculated from the expressi

$$M_e = \frac{2RT \cdot \ln (C_2/C_1)}{(1 - V\rho)\omega^2(X_2^2 - X_1^2)}$$

where C_1 and C_2 are the concentrations at the distances X_1 and X_2 fr the center of rotation. This equation has been shown to give the weig or Z-average for polydispersed material.[10]

The behavior of filamentous molecules in sedimentation equilibri measurements is not ideal. Attempts have been made to overcome t by taking into account the dependence of sedimentation and diffus constants on concentration[17] and also the deviations from ideal behav of each component in the mixture.[22]

Ultracentrifuge data can also be interpreted to give information ab the effective shape of the dissolved polymer molecules. The act frictional coefficient (f) can be calculated from the sedimentation c stant, and this compared with the theoretical figure (f_0) calculated fr Stokes' law (assuming a spherical molecule). The frictional ratio (f has been called the asymmetry coefficient. It expresses the deviation the molecule in solution from a spherical shape, and can be interpreted terms of the axial ratio of idealized particles.

1. *Molecular Weight Distributions*

No polysaccharides have yet been shown to be homogeneous molecular weight, and consequently the value of the experimenta determined average molecular weight depends upon the method us (Table 1). The three types of average obtained have been defined Lansing and Kraemer[10] as follows.

The *number-average molecular weight* is obtained through use of t formula

$$\bar{M}_n = \sum_i n_i M_i \Big/ \sum_i n_i$$

where n_i is the number of gram moles of molecular weight M_i, and t summation is taken over all values of i. $\bar{M}_n$ is thus the simple arithme

(22) M. Wales, F. T. Adler and K. E. van Holde, *J. Phys. & Colloid Chem.*, 145 (1951).

nean weight of the molecules, and is given by methods, such as the osmotic, which count the number of molecules present in solution.

The *weight-average molecular weight* follows from the formula

$$\bar{M}_w = \sum_i n_i M_i^2 \Big/ \sum_i n_i M_i = \sum_i c_i M_i \Big/ \sum_i c_i \qquad (12)$$

where c_i is the concentration of the i-th species. $\bar{M}_w$ is thus an average of weight fractions $\left(c_i \Big/ \sum_i c_i\right)$ each weighted by their molecular weight, and therefore it emphasizes the contribution of the higher molecular weights to the average. Light scattering and ultracentrifuge measurements result in this kind of average, and if Staudinger's rule is obeyed, so do measurements of intrinsic viscosity. If equation (6) holds then the average depends upon the value of (α), and a viscosity molecular weight average $\bar{M}_v$ may be defined as:

$$\bar{M}_v = \left(\sum_i n_i M_i^{\alpha+1} \Big/ \sum_i n_i M_i\right)^{1/\alpha} \qquad (13)$$

The *Z-average molecular weight* follows from the formula

$$\bar{M}_z = \sum_i n_i M_i^3 \Big/ \sum_i n_i M_i^2 \qquad (14)$$

This is a purely mathematical average which emphasizes the higher molecular weights even more, and results from one method of evaluation of sedimentation and diffusion measurements. Higher moment averages can also be obtained, but with low experimental accuracy, and they are therefore of small importance.

For a homogeneous substance all the above averages are equal; for polydisperse substances $\bar{M}_z > \bar{M}_w > \bar{M}_n$. The extent to which the averages differ from each other is a measure of the polydispersity of the substance.[23]

Only after a complete determination of the molecular weight distribution of the substance can it be said to be fully characterized. This is, however, a difficult task which would appear best approached by intensive fractionation of the material.

III. Assessment of Methods

The choice of method must depend upon the exact nature of the problem to be solved. Methods providing a number-average are clearly

(23) For the relationship between different molecular weight averages see for example M. F. Bechtold, *J. Polymer Sci.*, **4**, 401 (1949).

necessary for determining the degree of branching of a molecule after estimation of end-groups by chemical assay, and for following degra tion or synthesis where the number of bonds broken or formed is requir The osmotic method is probably the most suitable for this purp Viscometry also may be used, provided it can be properly calibrated osmometry. It then has considerable advantages in quickness a sensitivity.

The ultracentrifuge gives the clearest indication of the polydisper of a polymer, and in conjunction with diffusion measurements it provi a reliable weight-average.

Too few measurements of polysaccharides have yet been made light-scattering to assess its value for these polymers, but it may inferred from work carried out on other systems that it has considera potentialities.

Clearly as many methods as possible should be combined to obt complete characterization of a polysaccharide.

IV. Problems Inherent in Physico-chemical Studies of Polysaccharides

In addition to the types of information mentioned in the Introd tion, the complete characterization of a polysaccharide requires a kno edge of (1) the general structure of the molecules, (2) the differ molecular weight-averages and the molecular weight-distribution, and the shape of the molecules.

Organic-chemical studies cannot determine completely the struct of polysaccharides, as such methods can only account for about 95% the linkages present. In many instances there is evidence that lab bonds exist, and the presence of these can only be determined from kine studies of the rates of hydrolysis of the polysaccharide under differ conditions. The establishment of the presence or absence of such lab linkages should form an important preliminary in the study of polysaccharides, especially as the breakdown occurring during isolati may be of this nature.

As the unsubstituted polysaccharides are rarely stable in solution, so ble derivatives must almost invariably be used, and a study of the u voidable degradation accompanying the preparation of such derivative also essential. The use of soluble derivatives may not be entirely sat factory as there is the possibility that linear natural polymers mi aggregate in solution (compare the behavior of certain synthetic polym *e.g.*, polyvinyl chloride[24]). A similar effect may occur in the presence

(24) P. Doty, H. Wagner and S. Singer, *J. Phys. & Colloid Chem.*, **51**, 32 (19

traces of inorganic ions, as has been found in the case of cellulose derivatives.[25] It is not easy to prove experimentally the existence of physical aggregation, and little work on this problem in the field of polysaccharides has yet been attempted.

V. The Molecular Weights of Polysaccharides Containing One Type of Structural Unit

1. *Xylans*

The only pentosans which have been studied to any great extent are the xylans. These can be obtained from all lignified cell membranes by extraction with 5% sodium hydroxide solution after lipids, pectic materials etc. have been removed. The conditions necessary to avoid degradation during isolation have been outlined.[26] Early structural determinations indicated that arabinose was present as a non-reducing end-group in a branched molecule which had a chain-length of 18–20 D-xylofuranose residues.[27] It has been shown recently, however, that the xylan from esparto holocellulose can be purified free of arabinose after several re-precipitations of the copper-complex.[28]

The chain-length of this arabinose-free xylan was shown by methylation and estimation of the hydrolysis products, using chromatographic methods, to be 35 ± 3 D-xylose units. Osmotic pressure and viscosity measurements indicated that the degree of polymerization of the methylated and acetylated derivatives was about 70–90 xylose residues, and a structure for the xylan involving branching was confirmed.

The molecular weights of straw and beech xylans have been studied by Husemann,[29] who claimed that the preparation of certain derivatives was accompanied by little degradation. The size of the products was determined by osmotic pressure measurements, and in the case of straw xylan, the original material, having a degree of polymerization (D. P.) of 150 xylose residues, was converted relatively undegraded to the methyl ether (D. P. = 113), and then to the methyl-acetyl derivative (D. P. = 123). Beech xylan behaved similarly; the original material, having a degree of polymerization of 150, was converted unchanged into

(25) H. Campbell and P. Johnson, *J. Polymer Sci.*, **3**, 735 (1948); M. Wales and D. L. Swanson, *J. Phys. & Colloid Chem.*, **55**, 203 (1951).

(26) R. L. Whistler, *Advances in Carbohydrate Chem.*, **5**, 269 (1950).

(27) R. A. S. Bywater, W. N. Haworth, E. L. Hirst and S. Peat, *J. Chem. Soc.*, 1983 (1937).

(28) S. K. Chanda, E. L. Hirst, J. K. N. Jones and E. G. V. Percival, *J. Chem. Soc.*, 1289 (1950).

(29) E. Husemann, *Naturwissenschaften*, **27**, 595 (1939); *J. prakt. Chem.*, **155**, 13 (1940).

the benzoyl-acetyl derivative (D. P. = 147). Fractionation, which followed viscometrically, showed that both xylans were relatively ho geneous, straw xylan containing 90%, and beech xylan 70%, of molec of the same size. As the value of K_m in Staudinger's equation was fo to be identical with that for cellulose, it was suggested that the mole was linear.

Further studies would appear to be necessary before the result chemical and physical determinations can be satisfactorily correla and few estimates of the molecular size have been made.

2. *Glucosans (with the Exception of Starch and Cellulose)*

a. Glycogen.—This polysaccharide, which resembles amylopectin usually prepared under rather drastic conditions by treating the sou (usually certain animal organs) with strong alkali or trichloroacetic a to remove associated proteins. It possesses a highly branched struct of D-glucose units joined along the chains by α-1,4-glycopyranos bonds. The nature of the cross-linkages is not yet known with c tainty.[30] Glycogens were thought to have an average chain-length either 12 or 18 anhydroglucose units depending on the source, but it now been claimed that all possess a chain-length of 12 units.[31]

A large number of molecular weight measurements have been m on glycogens from various sources. The natural material, in contr to the behavior of starch fractions, appears to be stable in aqueous so tion, and measurements have been made on the unsubstituted polys charide as well as on soluble derivatives.

The first molecular weight measurements appear to be the osm pressure determinations carried out by Oakley and Young[32] on uns stituted rabbit liver and muscle glycogens in 0.1 *N* calcium chloride so tion. They claimed that products obtained by alkaline extraction, by water extraction followed by precipitation by acetic acid, had same molecular size. The molecular weight of rabbit liver glycogen found to be $(1.2–2.2)10^6$, and that of muscle glycogen $(0.7–1.8)10^6$.

Solutions of the glycogens were also found to exhibit a higher osm pressure when salt-free than in the presence of calcium chloride. It suggested that this behavior, which is surprising in view of the absenc ionizable groups in glycogen, was due to Donnan effects. Sim osmotic pressure measurements by Staudinger and Husemann[33] on n

(30) D. J. Bell, *J. Chem. Soc.*, 992 (1948); K. H. Meyer and M. Fuld, *Helv. C Acta*, **24**, 375 (1941).

(31) M. Abdel-Akher and F. Smith, *J. Am. Chem. Soc.*, **79**, 995 (1951).

(32) H. B. Oakley and F. G. Young, *Biochem. J.*, **30**, 868 (1936).

(33) H. Staudinger and E. Husemann, *Ann.*, **530**, 1 (1937).

degraded glycogen samples did not show any dependence on salt concentration, however, and one sample that was measured in water, in 0.1 *N* calcium chloride and in formaldehyde possessed an apparent molecular weight of 283,000 (1750 hexose units) in all three solvents. In certain instances this dependence of physical properties on salt concentration has recently been confirmed by osmotic pressure[34] and light-scattering determinations.[35]

Bell and co-workers[34] confirmed the lowering of osmotic pressure in the presence of salts as observed by Oakley and Young.[32] These authors suggested that as disaggregation and the Donnan effect were unlikely, an explanation might be that the salt caused a decreased asymmetry in the molecule with a resultant entropy change.

By analogy with starch fractions, it seemed possible that the presence of phosphate groups might influence the behavior of glycogen. This was investigated by Putzeys and Verhoeven,[35] who when measuring the molecular weight of commercial samples of glycogen by the light-scattering method found that the apparent molecular weight depended upon the concentration of glycogen and the ionic strength of the solvent. This effect did not occur with carefully prepared and purified glycogen samples. Phosphorylation was found to have no effect on the apparent molecular weight. It was therefore suggested that traces of protein material, or carboxyl groups caused by treatment with alkali, were most likely the cause, and that all glycogens described in the literature which showed such an anomalous effect were either degraded or impure.

The polydispersity of glycogen preparations in the ultra-centrifuge was shown by Mystkowski,[36] who was examining protein–glycogen interactions. Bridgman[37] first measured the sedimentation and diffusion constants of rabbit liver glycogen preparations. Molecular weights were found to be of the order of $(4)10^6$ ($S_{20} = (65\text{–}82)10^{-3}$: $D_{20} = (1.1)10^7$) for various preparations. All the products were inhomogeneous, and it was not possible to prove whether the results indicated aggregation or a true molecular weight. Again there was little difference in the size of the products from alkaline and acidic extractions. The frictional ratio ($f/f_0 = 1.9$), indicated that the molecule was not spherical, but elongated in solution. One sample of glycogen had a molecular weight of $(13.9)10^6$ ($S_{20} = (100)10^{-13}$: $D_{20} = (1.1)10^7$).

A glycogen of very high molecular weight has been isolated from avian

(34) D. J. Bell, H. Gutfreund, R. Cecil and A. G. Ogston, *Biochem. J.*, **42,** 405 (1948).

(35) P. Putzeys and L. Verhoeven, *Rec. trav. chim.*, **68,** 817 (1949).

(36) E. M. Mystkowski, *Biochem. J.*, **31,** 716 (1937).

(37) W. B. Bridgman, *J. Am. Chem. Soc.*, **64,** 2349 (1942).

tubercle bacilli by Chargaff and Moore.[38] Sedimentation and diffus measurements showed the molecular weight to be (12–13)10^6 (page 326).

The molecular weights of glycogens from different sources h recently been compared, using sedimentation and osmotic pres measurements.[34] A significant difference was found in the size of products from different sources (Table II), but although all samp

TABLE II

Sedimentation and Diffusion Data for Glycogen Samples[34]

Source	$\frac{\text{Specific viscosity}^a}{\text{Concentration}}$	$S_{20}(10)^{13}$ *in normal NaCl*	$D_{20}(10)^{7}$ *in normal NaCl*	*Molecu weigh*
Horse muscle[b]	0.074	63	1.50	2.9(10
Rabbit muscle[b]	0.074	58	1.55	2.6(10
Human muscle[b]	0.060	57	1.65	2.4(10
Rabbit liver[b]	0.065	81	1.27	4.4(10
Rabbit liver[c]	0.063	75	1.21	4.3(10
Ascaris lumbricoides[c]	0.135	18	1.80	0.70(10

[a] Measured in water.
[b] Extracted by NaOH solution.
[c] Extracted by water.

were polydisperse there was general uniformity among samples from a one source. In agreement with Bridgman,[37] liver glycogens were fou to have molecular weight values of (4.3–4.4)10^6, alkaline extracti having little effect. Muscle glycogens were found to have values (2.4–2.9)10^6, whilst the glycogen from the whole tissues of *Asca lumbricoides* had a much smaller molecular weight of (0.7)10^6. T estimated molecular weights from the osmotic pressure determinatio were only approximate, as extrapolation was difficult. The salt effect osmotic measurements that had been found by Oakley and Young[32] w confirmed.

A colorimetric method has been used by Meyer[39] to determine t size of fractions of degraded liver glycogen. A glycogen having molecular weight of 290,000 was separated by electrodecantation into soluble fraction whose value was 95,000, and an insoluble fraction whe value was 320,000.

Recent determinations using the light-scattering method[35,40] ha given results which indicate larger particle sizes. Putzeys and V

(38) E. Chargaff and D. H. Moore, *J. Biol. Chem.*, **155**, 493 (1944).
(39) K. H. Meyer, G. Noelting and P. Bernfeld, *Experientia*, **3**, 370 (1947).
(40) H. Staudinger, *Makromol. Chem.*, **2**, 88 (1948).

hoeven,[35] using carefully prepared glycogen samples, found a value of $13.8(10)^6$ for the molecular weight of rabbit muscle glycogen, and for two fractions of rabbit liver glycogen values of $6.3(10)^6$ and $18.5(10)^6$. The apparent molecular weight was independent of both the glycogen concentration and ionic strength. Very high values were also found by Staudinger.[40] He claimed that the molecular weight of the glycogen depended upon the source; for muscle glycogens from various animal species values of $1.5(10)^6$ were found, and for the corresponding liver glycogens values of up to $23(10)^6$, whilst heart glycogens from the cat and pig were smaller, having values of $1(10)^6$ and $2(10)^6$.

Fewer molecular weight measurements on derivatives of glycogen have been made. Staudinger[33] examined the effect of acetylation on molecular size and showed that this esterification process involved no degradation; *e.g.*, the degree of polymerization of a glycogen sample was 1750, that of the corresponding triacetate 1680, and that of the regenerated glycogen 1730. Oakley and Young[32] claimed that methylation caused little degradation, but the measurements of Carter and Record[41] do not support this view. These workers measured the osmotic pressure of methylated and acetylated liver glycogens from various sources (Table III), and found a smaller molecular size for the methylated derivative. (The osmotic measurements on the acetylated derivatives were carried out at the limiting sensitivity of the apparatus.)

TABLE III

Osmotic Pressure Determinations on Glycogen Derivatives[41]

Source	*Derivative*	*Molecular weight*	*D. P.*
Rabbit liver	Methyl ether	$0.62(10)^6$	3,000
	Acetate	$2.5(10)^6$	8,700
Dog fish liver	Methyl ether	$0.62(10)^6$	3,000
	Acetate	$3.5(10)^6$	12,000
Haddock liver	Methyl ether	$0.62(10)^6$	3,000
	Acetate	$1.3(10)^6$	4,500
Hake liver	Methyl ether	$0.27(10)^6$	1,340
	Acetate	$1.9(10)^6$	6,600

Record[42] later studied the same methylated samples in the ultracentrifuge. As expected from the polydisperse nature of all the samples, the molecular weight values ($1.90(10)^6$, $3.58(10)^6$, $0.97(10)^6$ and $1.13(10)^6$ for the derivatives in Table III) were higher than the corresponding

(41) S. R. Carter and B. R. Record, *J. Chem. Soc.*, 660 (1939).

(42) B. R. Record, *J. Chem. Soc.*, 1567 (1948).

values from osmotic pressure measurements. The asymmetric natu molecules in solution was shown by the frictional ratio ($f/f_0 = 1.72$-2 Although Record suggested that methylation and acetylation inv little degradation, these values appear to be smaller than those obt from measurements on the corresponding free glycogens.

A molecular weight of $6.1(10)^6$ has been recorded for the acet mollusc muscle glycogen by Meyer and Jeanloz[43] on the basis of osm pressure determinations. Fractions obtained from this glycoge precipitation with methanol possessed values for the molecular weig $6.1(10)^6$, $2.1(10)^6$, and $3.0(10)^6$. It was suggested that these repres minimum values because of the effect of impurities of small mole weight.

All glycogen samples appear to be polydisperse, and most poss high molecular weight of the order of $2(10)^6$ at least, although the depends on the method of extraction. A considerable amount of dence would suggest that the molecular weight of the glycogen depends on the source (see Tables II and IV). The anomalous phy chemical behavior found for certain specimens examined may be du either protein impurities or to artifacts formed during isolation. direct proof appears to exist, however, that the molecules are m disperse in aqueous solution, particularly as glycogens can sometim separated into soluble and insoluble fractions by physical means. dence might be obtained on this point by a study of derivatives of samples of very high molecular weight.

Ultracentrifuge measurements indicate that the glycogen mole in solution is not spherical, but possesses an elongated form.

b. Lichenin.—This polyglucose is easily extracted with hot w from Iceland moss (*Cetraria islandica*). It has been shown that a 30% of the units in the polysaccharide are joined by α-1,3- and remainder by α-1,4-glycosidic linkages.[44]

The unbranched nature of the molecule was established by Co and Record.[41] These workers found the molecular weight of the met ated derivative determined by osmotic pressure measurements to b the same order as that calculated from the percentage of end-grou methylation studies. This result has been confirmed by Meyer Gürtler,[44] who showed that the molecular weight of 26,000 (160 he units) calculated from a determination of reducing groups (usi colorimetric method) was in agreement with that from the analys non-reducing groups.

(43) K. H. Meyer and R. W. Jeanloz, *Helv. Chim. Acta*, **26**, 1784 (1943).
(44) K. H. Meyer and P. Gürtler, *Helv. Chim. Acta*, **30**, 751 (1947).

Molecular Weight Determinations on Glycogen Samples

Glycogen	*Source*	*Method of extraction*	*Derivative studied*	*Method*[a]	*Solvent*	*Molecular weight*	*Approx. degree of polymerization*	*Ref.*
Liver	Rabbit	Water & alkali	Unsubstituted	O. P.	0.1 *N* $CaCl_2$	$(1.18–2.16)10^6$	$11(10)^3$	32
	Rabbit	Water & alkali	Unsubstituted	S. & D.	0.1 *N* $CaCl_2$	$(4.6–5.2)10^6$	$28(10)^3$	37
	Rabbit	Water & alkali	Unsubstituted	S. & D.	*N* NaCl	$(4.3–4.4)10^6$	$24(10)^3$	34
	Rabbit	Water (hot)	Unsubstituted	τ	0.1 *N* KCl	$(6.3–18.5)10^6$	$25(10)^3$	35
	Rabbit	Water (hot)	Unsubstituted	τ	0.1 *N* KCl	$23(10)^6$	$130(10)^3$	40
	Rabbit	Alkali	Methyl ether	O. P.	$CHCl_3$	$0.62(10)^6$	$3.0(10)^3$	41
		Alkali		S. & D.		$1.90(10)^6$	$1.0(10)^3$	42
	Rabbit	Alkali	Acetate	O. P.	$CHCl_3$	$2.5(10)^6$	$8.7(10)^3$	41
	Fish	Alkali	Methyl ether	O. P.	$CHCl_3$	$0.6(10)^6$	$3.3(10)^3$	41
		Alkali	Acetate	O. P.	$CHCl_3$	$(1.9–3.5)10^6$	$(1.0–1.9)10^3$	41
Muscle	Rabbit	Alkali (hot)	Unsubstituted	S. & D.	*N* NaCl	$2.6(10)^6$	$14(10)^3$	34
	Rabbit	Water (hot)	Unsubstituted	τ	0.1 *N* KCl	$13.8(10)^6$	$8(10)^3$	35
	Rabbit	Alkali (hot)	Unsubstituted	τ	0.1 *N* KCl	$1.5(10)^6$	$77(10)^3$	40
	Mollusc	Water (hot)	Acetate	O. P.	$PhCH_2OH$	$(2.1–6.1)10^6$	$(12–34)(10)^3$	43
	Horse	Alkali (hot)	Unsubstituted	S. & D.	*N* NaCl	$2.9(10)^6$	$16(10)^3$	34
	Human	Alkali (hot)	Unsubstituted	S. & D.	*N* NaCl	$2.4(10)^6$	$10(10)^3$	34
Heart	Pig	Alkali (hot)	Unsubstituted	τ	*N* NaCl	$2.0(10)^6$	$11(10)^3$	40
	Cat	Alkali (hot)	Unsubstituted	τ	*N* NaCl	$1.0(10)^6$	$5.5(10)^3$	40
Ascaris lumbricoides	Whole tissues		Unsubstituted	S. & D.	*N* NaCl	$0.70(10)^6$	$3.9(10)^3$	34
Mycobacterium tuberculosis	Avian	Borate buffer	Unsubstituted	S. & D.	Buffer	$(12–13)10^6$	$(67–72)10^3$	38

[a] O. P. = osmotic pressure measurement: S. & D. = sedimentation and diffusion measurement: τ = turbidity measurement.

Determinations were also made by Carter and Record[41] on a derivatives of lichenin, and a comparison of the results with tho the methylated derivative (Table V) shows that degradation must occurred during methylation, although this was carried out vi acetate.

TABLE V

Osmotic Pressure Determinations on Derivatives of Lichenin[41]

Derivative	*Solvent*	*Molecular weight*	*D. P.*	*End-g assay o methyl*
Lichenin acetate (A)	Chloroform	118,000	410	–
Lichenin acetate (B)	Chloroform	36,500	127	–
Lichenin methyl ether (from A)	Carbon tetrachloride	10,700	52	*ca.*
Lichenin methyl ether (from *B*)	Carbon tetrachloride	13,900	68	–
Directly methylated lichenin	Chloroform	33,100	162	–

Although lichenin has been shown to possess an unbranched ture, further investigations, particularly of the degradation occu during the preparation of derivatives, appear to be necessary to d mine the molecular size of the polysaccharide in its native state.

c. Laminarin.—Laminarin, which is found in certain common br seaweeds, is another polyglucose. It is liberated as an insoluble pr tate from *Laminaria claustoni* when the fronds are leached in aci water. Methylation studies have shown the average chain-length o polysaccharide to be 20 D-glucose residues, the units being joine β-1,3-glycosidic linkages.[45] The laminarin from *L. digitata* diffe that a precipitant such as ethanol is necessary before it is liberated has been suggested that this behavior is a function of molecular size, this form of laminarin was investigated by Percival and Ross[46] to d mine whether there were any structural differences between the polysaccharides. Although 3% of fucoidin was present in the lamin from *L. digitata*, no other structural differences were found, and chain-length was again about 20 units. On X-ray examination, methyl- and acetyl-derivatives of the two forms were indistinguish and the molecular weights of the methylated laminarins, as determ by use of a modified Barger's method, were of the same order (equiv

(45) V. C. Barry, *J. Chem. Soc.*, 578 (1942); J. J. Connell, E. L. Hirst and E. Percival, *ibid.*, 3494 (1950).

(46) E. G. V. Percival and A. G. Ross, *J. Chem. Soc.*, 720 (1951).

14–18 hexose units). It would consequently appear that the laminarin om both seaweed sources is an unbranched polyglucose having a chain-ngth of about 20 units.

d. Bacterial Dextrans.—Polyglucoses are synthesized by *Leuconostoc esenteroides* (utilizing a pentose medium) and by *Leuconostoc dextranicum* (utilizing a sucrose medium). There is a great difference in the verage chain-length of dextrans from the two sources; the values for hose produced by various strains of *L. mesenteroides* have been found to ary from 3 to 24 units[47,48] whereas those from *L. dextranicum* are of the rder of 200–500 units.[49,50]

Degraded dextrans from *L. mesenteroides* (when dissolved in physiological saline) have found important use as a blood plasma extender. n investigation of the molecular weight of these polysaccharides has een carried out by Ingelman and Halling[51] as for this purpose the olecular size of the dextran must be of the same order as that of the lasma proteins. They studied fractionated dextrans in the ultra-entrifuge. The fractions obtained were not homogeneous and both the edimentation and the diffusion constants were dependent upon the oncentration. Values of molecular weight ranged between 14,000 and 8,000,000 (Table VI), and for all fractions the value of the frictional atio ($f/f_0 > 1$) indicated that the particles were non-spherical. (This grees with electron-microscope studies.)[52] For the molecular weight

TABLE VI

The Molecular Constants of Some Dextran Fractions[51]

(Partial specific volume (V) = 0.62. Solvent: phosphate buffer)

Fraction	*Intrinsic viscosity*	$S_{20}(10)^{13}$	$D_{20}(10)^{7}$	f/f_0	*Molecular weight*	*Approx. D. P.*
J IV	0.090	1.64	7.50	1.88	14,000	80
J I	0.18	2.64	4.69	2.19	36,000	200
I V	0.25	4.10	3.12	2.49	84,000	450
I IV	0.29	5.30	2.40	2.72	141,000	800
I III	0.38	6.55	1.73	3.15	240,000	1,300
II, 10a,β	0.74	23.3	0.75	3.61	1,990,000	11,000
II, 10a,α	0.86	26.4	0.55	4.25	3,050,000	17,000
II 2a	1.26	150.0	0.25	4.03	38,000,000	210,000

(47) Allene Jeanes and C. A. Wilham, *J. Am. Chem. Soc.*, **72**, 2655 (1950).
(48) T. H. Evans and H. Hibbert, *Advances in Carbohydrate Chem.*, **2**, 203 (1946).
(49) S. Peat, Elsa Schlüchterer and M. Stacey, *J. Chem. Soc.*, 581 (1939).
(50) M. Stacey and G. Swift, *J. Chem. Soc.*, 1555 (1948).
(51) B. Ingelman and M. S. Halling, *Arkiv Kemi*, **1**, 61 (1949).
(52) B. Ingelman and K. Seigbahn, *Nature*, **154**, 237 (1944).

range suitable for use as a blood plasma extender (40,000–300,000 relationship

$$[\eta] = 8.2(10)^{-7}\ M + 0.18$$

was found. The curve of *ln.* M as a function of *ln.* $[\eta]$ for the whole of molecular weights was non-linear. The large linear portion (40,0 $M < 2{,}000{,}000$) had a very low value[53] for the slope of $\alpha = 0.34$, could not be explained.

The units in the polyglucose synthesized by *L. dextranicum* have shown to be joined by α-1,6-glycosidic linkages.[54] The results chemical end-group assay on the methylated dextran indicate average chain-length of about 500 D-glucose residues,[49,50] and as os pressure measurements on the same derivative gave a molecular wei 44,000 (200 hexose units), the molecule was considered to be unbran

A considerably smaller value for the molecular weight of the m ated product of this dextran has been obtained by Hassid and Ba From viscosity measurements (using $K_m = (10)^{-3}$ for methyl cellul the molecular weight was roughly indicated as 11,700, whilst the from sedimentation equilibrium measurements was 3,275 (althoug latter figure was not considered very reliable).

With the exception of dextrans from *L. mesenteroides*, very would appear to be known of the molecular size of bacterial polyglu

e. Other Polysaccharides Resembling Dextrans.—Betabacterium formé (Ward-Mayer) produces a polyglucose shown by methy studies[56] to possess an average chain-length of 25 glucose resid which the units are joined by α-1,6-glycosidic linkages. Osmotic sure determinations on the methylated derivative were found to in a molecular weight of about 101,000 (500 units), while an average length of 27 was obtained from estimations of the iodine number unsubstituted polysaccharide. These results suggest that the mo possesses a branched structure.

A water-soluble polysaccharide of low molecular weight produc *Phytomonas tumefaciens* (crown-gall organism) is a polyglucose in the units are joined by β-1,2-glycosidic linkages.[57] Sedimentation diffusion measurements[58] indicated a molecular weight of 3600

(53) The value of α in the modified Staudinger equation $[\eta] = KM^{\alpha}$ (see pag

(54) W. Z. Hassid and H. A. Barker, *J. Biol. Chem.*, **134,** 163 (1940).

(55) H. Staudinger and F. Reinecke, *Ann.*, **535,** 47 (1938).

(56) W. D. Daker and M. Stacey, *J. Chem. Soc.*, 585 (1939).

(57) E. W. Putnam, A. L. Potter, R. Hodgson and W. Z. Hassid, *J. Am. Soc.*, **72,** 5024 (1950).

(58) F. C. McIntire, W. H. Peterson and A. J. Riker, *J. Biol. Chem.*, (1942).

$S_{20} = 1.2(10)^{-13}$: $D_{20} = 2.11(10)^{-6}$: V = 0.61), which is equivalent to 2 glucose units per molecule.

f. Yeast Insoluble Polysaccharide.—The skeletal polysaccharide of yeast has been shown to be a polyglucose in which the residues are joined by 1,3-glycosidic linkages.[59] An indication that the molecular weight was of the order of 8,000 (36 units) was obtained from the specific viscosity, and a closed-chain type of structure for the molecule was suggested as no end-groups were found in the methylation studies.

Periodate oxidation,[60] however, would indicate that the material is highly branched with an average chain-length of 9 units. A detailed knowledge of the structure and size of this polysaccharide is lacking.

3. *Galactans*

Galactans are found in association with other hemicelluloses in deciduous and coniferous woods, certain seeds and pectins. It is very difficult to obtain the pure polysaccharide. Not much is known of the detailed structure of these molecules,[61] and few molecular weight measurements have been carried out.

The water-soluble product designated ϵ-galactan from larch has been shown from the sedimentation diagrams obtained in the ultracentrifuge to consist of two nearly mono-dispersed components,[62] an arabogalactan of molecular weight 16,000 and a β-galactan of molecular weight 100,000. These two components were found to be present in the ratio of 1:4. The resolution of this mixture of polysaccharides has been confirmed in methylation studies, which also showed the β-galactan to have a highly branched structure.[63]

The above structure is very different from that of the galactan associated with pectin in the seeds of *Lupinus albus.* This was shown to have a chain-length of 120 units.[64] As no molecular weight measurements have been carried out on this material, it is not known whether the molecule is linear or branched.

4. *Mannans*

Mannans are found more often in coniferous than in deciduous woods, and they also occur in the thickened cell walls of hard seeds, *e.g.*, the

(59) W. Z. Hassid, M. A. Joslyn and R. M. McCready, *J. Am. Chem. Soc.*, **63**, 295 (1941).

(60) D. J. Bell and D. H. Northcote, *J. Chem. Soc.*, 1944 (1950).

(61) See E. L. Hirst and J. K. N. Jones, *Advances in Carbohydrate Chem.*, **2**, 235 (1946).

(62) T. Svedberg, *J. Phys. and Colloid Chem.*, **51**, 1 (1947); T. Svedberg and H. Mosimann; *Kolloid Z.*, **100**, 99 (1942).

(63) W. G. Campbell, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 774 (1948).

(64) E. L. Hirst, *J. Chem. Soc.*, 70 (1942).

ivory nut (*Phytelepas macrocarpa*). They are closely bound cellulosic materials, and are difficult to purify except by treatment strong alkali. The detailed structures of few of these polysacch are known, although by methylation studies the mannan from ivor has been shown to possess a chain-length of 80 hexose residues,[65] by β-1,4 linkages.

The mannans from spruce and pine, and from salep (*Tubera* have been examined by Husemann,[29,66] who followed the changes ring during the preparation of several derivatives by measurin molecular weight of the products by osmotic pressure determina No degradation was found during the formation of the acetyl-, ben and nitro-derivatives; as in the case of the spruce and pine mannan original degree of polymerization of 160 was unchanged. The va K_m in Staudinger's equation for the unsubstituted mannans in Schwe reagent was found to be $4.4(10)^{-4}$, the same as for cellulose. Fract tion and a study of the viscosity of the fractions showed both these nans to be fairly homogeneous. It was deduced from the visc measurements that the molecule was linear. Salep mannan, which been isolated with water to avoid degradation, was found to be inh geneous. Fractions were obtained with molecular weights for the polysaccharides of 27,300–220,000 (164–1340 hexose units), which unaltered when the polysaccharide fractions were nitrated. value for K_m was again found to be $4.4(10)^{-4}$ for the free mann Schweizer's reagent. These results would indicate that these man have unbranched molecules, with a degree of polymerization of 200 units.

A very different structure has been found for the mannan th present in yeast. Methylation and hydrolysis[67] showed this to highly branched molecule. The methylated material was inh geneous, the intrinsic viscosity of different fractions varying bet 0.062 and 0.212, with corresponding molecular weights (determine osmotic pressure measurements) between 18,000 and 76,000 (90 hexose units).

5. *Fructosans*

Polyfructoses are widely distributed as reserve food materia plants. Usually these water-soluble polysaccharides can be extr from their sources (*e.g.*, tubers) without use of drastic reagents, since are found in solution in cell sap.

(65) F. Klages, *Ann.*, **509**, 159 (1934), **512**, 185 (1934).
(66) E. Husemann, *J. prakt. Chem.*, **155**, 241 (1940).
(67) W. N. Haworth, R. L. Heath and S. Peat, *J. Chem. Soc.*, 833 (1941).

a. Inulin.—This polysaccharide, which is found in tubers of *Compositae*, can be isolated from the aqueous extract by precipitation with ethanol. Although originally it was thought that the molecule was a straight chain of about 30 D-fructose units joined by 1,2 glycosidic linkages, recent work suggests that D-glucose (6%) is incorporated into the inulin structure.[68]

A molecular weight determination on inulin acetate, using Rast's method, showed the molecular weight to be 6300 (22 units).[69] Carter and Record[41] have compared the molecular weight of the methylated derivative determined by osmotic pressure measurements with the results of an end-group assay. The molecular weight was found to be 5610 (30 hexose units), and as the chain-length was also 30 units, the molecule was shown to be linear. A similar determination of the molecular weight of acetylated inulin have a value of 8880 (30 units).

Inulin would therefore appear to be an unbranched molecule consisting of about 30 D-fructose units, although the position regarding the glucose molecules which may be part of the structure is not definite.

b. Other Fructosans.—Many other fructosans have been found in plants.[70] A large number have been studied by Schlubach, who believes that, as a rule, fructosans are made up of D-fructose residues joined in a long. The molecular weights of those shown in Table VII are based on cryoscopic measurements by Schlubach. As such measurements are not very satisfactory for large molecules, the correct values are probably higher than quoted.

TABLE VII

The Molecular Weights of Polyfructoses[70]

Fructosan	*Derivative*	*Molecular weight*	*D. P.*
Asparagosin	Unsubstituted	1635	9
	Acetate	3918	13–14
Phlegmistrin	Acetate	9517	*ca.* 33
Graminin	Unsubstituted	976	5–6
	Acetate	2700	9–10
Triticin	Unsubstituted	2600	14–15
Inulin	Methyl ether	3280	15–16

c. Levans (Bacterial).—Polyfructoses are synthesized by the action of a variety of aerobic organisms, including plant pathogens. Methyla-

(68) E. L. Hirst, D. J. McGilvray and E. G. V. Percival, *J. Chem. Soc.*, 1297 (1950).

(69) W. N. Haworth and H. R. L. Streight, *Helv. Chim. Acta*, **15**, 609 (1932).

(70) See: Emma J. McDonald, *Advance in Carbohydrate Chem.*, **2**, 253 (1946).

TABLE VIII

The Molecular Weight of Some Polysaccharides Containing One Type of Structural Unit

Polysaccharide	*Derivative studied*	*Method*[a]	*Solvent*	*Molecular weight*	*Degree of polymerization*	*Result from end-group assay*	*Structure*	*Reference*
Glucosans (excepting glycogen)								
Lichenin	Unsubstituted	Colorimetric	—	26,000	160	160	Linear molecule	44
	Methyl ether	O. P.	CCl_4	118,000	410	—		41
	Acetate	O. P.	$CHCl_3$	10,700	52	*ca.* 80		41
Laminarin	Methyl ether	Modified Barger's method	$CHCl_3$	$(2.9–3.8)10^3$	14–18	20	Linear molecule	46
	Acetate		$CHCl_3$	$(4.0–5.2)10^3$	14–18	20		46
Bacterial dextrans from:								
(1) *L. dextranicum*	Methyl ether	O. P.	$CHCl_3$	44,000	200	500	Linear molecule	49
	Methyl ether	Viscosity	*m*-Cresol	11,700(?)	50	None found		54
		Sedimentation equilibrium		3,275(?)	17			54
(2) *L. mesenteroides*	Unsubstituted	—	—	—	—	3–24	Branched(?)	47, 48
	Unsubstituted	S. & D.	Phosphate buffer	$14,000–(38)10^6$	80–210,000	—		51
(3) *B. vermiformé*	Methyl ether	O. P.	$CHCl_3$	101,000	500	27	Branched	56
(4) *P. tumefaciens*	Methyl ether	Sedimentation equilibrium		3,600	22	—	?	58
Yeast insoluble-polysaccharide	Methyl ether	Viscosity	*m*-Cresol	8,000(?)	36	—	Closed chain	59
Galactans								
Larch galactan	Unsubstituted	S. & D.	Water	100,000	550	—	Branched	62
Fruit pectin galactan	Methyl ether	—	—	—	—	120	?	64
Mannans								
Spruce and pine mannans	Unsubstituted	O. P.	0.1 *N* $CaCl_2$	29,000	160	—	Linear	29
	Acetate	O. P.	$CHCl_3$	26,500	147	—		29
Salep mannan	Nitrate	O. P.	Acetone	$(46–336)10^3$	167–1,220	—		66
	Unsubstituted	O. P.	Water	$(27.3–220)10^3$	164–1,340	—		66
Yeast mannan	Methyl ether	O. P.	$CHCl_3$	$(18–76)10^3$	90–380	—	Branched	67
Fructosans								
Inulin	Acetate	Rast		6,300	22	—		69
	Methyl ether	O. P.	$CHCl_3$	6,210	30	30	Linear	41
	Acetate	O. P.	$CHCl_3$	8,880	30	—		41
Levans:								
(1) Plant	Unsubstituted	Cryoscopic	—	1,000–3,000	5–20	—	Closed chain ?	70
(2) Bacterial	Unsubstituted	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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tion and hydrolysis studies have shown many to possess an average unit chain-length of 12.[48] Few physico-chemical measurements have been made but it has been suggested that the difference in physical properties of levans from different sources may be due to differences in molecular size.

The levan from *Bacillus vulgatus* has been studied in the ultracentrifuge by Ingelman and Seigbahn.[52] The values of the sedimentation and the diffusion constants obtained indicated molecular weights of 50–100 million ($S_{20} = (200–300)10^{-13}$: $D_{20} = 0.2(10)^{-7}$), these values being unaltered even if the levan was precipitated with alcohol, dried and redissolved.

Bacterial levans, as distinct from other polyfructoses, may be molecules possessing highly branched structures and very high molecular weights.

Table VIII summarizes the results of measurements of molecular weights of polysaccharides containing one type of structural unit.

6. *Polyuronides*

a. Pectic Materials.—Pectic materials are a group of polyuronides found in nearly all plant tissues. The tissues of fruits and fleshly roots are the most abundant sources of pectin, which is usually extracted from the powdered plant materials with acidified hot water or hot ammonium oxalate solutions, and then precipitated with ethanol. In most instances, pectins are difficult to prepare in a purified form, as they occur in association with arabans and galactans.[61] Their main component is pectic acid, which occurs naturally as the methoxylated derivative. Both pectins and pectic acid have been used for structural and molecular weight determinations. Pectic acid itself has been shown to consist of a chain of D-galacturonic acid residues joined primarily by α-1,4-glycosidic linkages.

The first probable values for the molecular size of the native pectins from apple and sugar beet were obtained by Schneider and co-workers.[71] These workers prepared the nitro- and acetyl-derivatives in order to avoid the anomalous behavior due to charge effects which occurred using the unsubstituted polysaccharides. From osmotic and viscometric measurements on the nitrate, values for the molecular weight of 30,000–100,000 were found, the constant K_m in Staudinger's equation being $6(10)^{-4}$. Viscometric measurements indicated that there was little difference in size between the two derivatives.

From measurements of the sedimentation and diffusion constants of

(71) G. Schneider and U. Fritschi, *Ber.*, **69**, 2537 (1936); G. Schneider and M. Ziervogel, *ibid.*, **69**, 2530 (1936); F. A. Henglein and G. Schneider, *ibid.*, **69**, 309 (1936).

pectins isolated directly from fruit juices, Svedberg and Gralén[72] rep... values for the molecular weight of 25,000–35,000 for apple, pear and ... pectins, and a higher value of 40,000–50,000 for orange pectin. ... products were polydisperse in the ultracentrifuge. Using the ... methods, Säverborn[73] found there was a difference between the mole... size of free pectins and those obtained by neutral or acidic extracti... higher temperatures. The products were inhomogeneous, and pe... from apple and citrus fruit albedo were found to have molecular we... of 50,000–100,000.

Säverborn[74] has also reported the results of investigation of ... physico-chemical properties of a large number of pectins. The ele... viscous effect shown by aqueous pectin solutions was eliminated in ... tions of high ionic strength. Certain ions (*e.g.*, La^{+++} and Th^{++++}) ... duced aggregation. Sedimentation and diffusion measurements ... also carried out in media containing a high concentration of univa... salts to eliminate similar charge effects. The apparent molecular we... depended upon the *p*H and the method of extraction (Table IX), the ... pectins isolated from the plant juices having a considerably higher v... for the molecular weight $(200–400)10^3$ than those extracted even ... mild reagents, which had molecular weights of about 60,000. All ...

TABLE IX

Molecular Constants of Some Pectins[74]

Source	*Solvent*	$S_{20}(10)^{13}$	$D_{20}(10)^{7}$	*Frictional ratio* (f/f_0)	*Molecular weight*
Free apple pectin	0.2 *N* NaCl	2.8	0.85	6.9	201,000
Commercial apple pectin	0.2 *N* NaCl	2.2	1.9	4.5	67,000
Pectic acid from apple pectin	0.2 *N* Na_2CO_3	2.0	2.0	4.3	62,000
Free citrus pectin	0.2 *N* NaCl	4.0	0.62	7.4	414,000
Extracted citrus pectin	0.2 *N* NaCl	2.2	1.5	5.1	89,000
Beet pectin	0.2 *N* NaCl	1.9	1.9	4.5	62,000
Red currant pectin	0.2 *N* NaCl	2.0	2.8	3.5	42,000
Carrot pectin	0.2 *N* NaCl	2.0	2.1	4.2	58,000
Flax pectin	0.2 *N* $(COONH_4)_2$	2.0	1.9	4.5	64,000

(72) T. Svedberg and N. Gralén, *Nature*, **142**, 261 (1938).

(73) S. Säverborn, *Kolloid Z.*, **90**, 41 (1940).

(74) S. Säverborn, "Contribution to the Knowledge of the Acid Polyuroni... Almqvist and Wiksells Boktryckeri AB, Uppsala (1945).

amples possessed a very elongated shape in solution ($f/f_0 > 1$). The alue of K_m from viscosity measurements was not consistent for different ectin samples, but a rough estimation of molecular weight could be btained by using the value of $10(10)^{-4}$. Sedimentation equilibrium easurements gave molecular weights which agreed with those from the ynamic measurements, and the polydispersity of the samples was there- ore shown to be not very great.

Viscosity measurements have provided the simplest method of ollowing the physico-chemical behavior of solutions of pectins. Maclay nd co-workers,[75] studying the viscometric behavior of citrus and apple ectins in 0.155 *N* sodium chloride as a function of *p*H and temperature, btained, using the expression $[\eta] = 1.4(10)^6(M)^{1.34}$, molecular weights f 23,000–71,000. The measurements indicated that the particles ehaved in solution as rigid rods. Osmotic pressure measurements were ound to give values for the number-average molecular weight of (1.8–3.9)10^4, and consequently the samples were considered inhomogeneous.

It has been shown recently that the maximum found in the η_{sp}/C versus C curve[76] when measuring the viscosity of solutions of sodium pectinate in sodium chloride, can be avoided when measuring the viscosity at different concentration, by varying the salt concentrations such that the total ionic strength of the solution remains constant.[77] It was suggested that this behavior could be utilized when the osmotic pressure or light-scattering determinations of pectic acid solutions were carried out.

Speiser and Eddy,[78] studying the effect of molecular size on gelling behavior, have determined the molecular weight of nitrated apple pectin fractions, and have so obtained the molecular weight distribution curve. The nitration procedure was claimed to cause little degradation. Values for the molecular weight of the fractions obtained from the nitrated native material were found to range between 32,000 and 213,000 (as calculated from viscosity measurements, using the data of Schneider and Fritschi[71]).

The molecular weight distribution curves for apple and lemon pectins were obtained similarly by Owens, Miers and Maclay.[79] These workers were not satisfied that the preparation of the nitrate involved no degrada-

(75) H. Lotzkar, T. H. Schulz, H. S. Owens and W. D. Maclay, *J. Phys. & Colloid Chem.*, **50**, 200 (1946); T. H. Schulz, H. Lotzkar, H. S. Owens and W. D. Maclay, *ibid.*, **49**, 554 (1945).

(76) D. T. F. Pals and J. J. Hermans, *J. Polymer Sci.*, **3**, 897 (1948).

(77) D. T. F. Pals and J. J. Hermans, *J. Polymer Sci.*, **5**, 733 (1950).

(78) R. Speiser and C. R. Eddy, *J. Am. Chem. Soc.*, **68**, 287 (1946).

(79) H. S. Owens, J. C. Miers and W. D. Maclay, *J. Colloid Sci.*, **3**, 277 (1948).

tion and they used the propionate for their studies. The prepar of this derivative was shown to be accompanied by only limited deg tion. The molecular weight distribution curves were obtained by tionating these derivatives and following the fractionation proce viscometrically. For a range of molecular weight of 20,000 to 150 the value of α in the modified Staudinger's equation[80] was found equal to 1.0 (the viscosity results being compared with those obt from osmotic pressure determinations on some of the fractions). relationship between the intrinsic viscosity and the molecular weight deduced as $M = 5.3(10)^4([\eta])$ for products having a high meth content, and $M = 4.6(10)^4([\eta])$ for those having a low methoxyl con

De-esterification of pectin by acid and enzyme action has been sh to be accompanied by degradation.[78] The same effect occurs on alk de-esterification, as was noted by Maclay and co-workers,[79] and as been studied by Vollmert.[81] It was shown from osmotic pres measurements that the alkaline de-esterification of pectin was ac panied by depolymerization even in an oxygen-free atmosphere. pectic acid itself was not affected similarly, Vollmert suggested that degree of polymerization depended upon the percentage of meth groups present in the polysaccharide. The values for the mole weights for the native apple and flax pectins of 140,000 and 100,000 to 50,000 and 80,000 respectively, when the substances were de-ester This behavior was thought to be due to the splitting of the acetal-lin of the galacturonic acid under the influence of the ester carbonyl gr

Esterification of the pectins with diazomethane at low temperat (less than −5°C.) was shown to cause little degradation[82] and the p ucts formed were suitable for viscometric determinations in aqu solution.

The pectins studied by Glikman and Orlov[83] would appear to been degraded, as osmotic pressure measurements indicated value the molecular weight of 4,000–33,000. These authors also found a linear relationship between the intrinsic viscosity and the mole weight, the most satisfactory equation being $[\eta] = 1.1(10)^{-5}(M)^{1.22}$.

Although the molecular size of the pectic substances depends large extent on the method of their isolation, the free substances w appear to possess high molecular weight of the order of 200,000 Table X). Studies of the degradation accompanying isolation and

(80) The value of α in the equation $[\eta] = KM^{\alpha}$ (see page 294).
(81) B. Vollmert, *Makromol. Chem.*, **5**, 110 (1950).
(82) B. Vollmert, *Macromol. Chem.*, **5**, 101 (1950).
(83) S. A. Glikman and S. I. Orlov, *Doklady Akad. Nauk., S.S.S.R.*, **71**, 895 (19

preparation of derivatives have been carried out and the presence of a labile linkage has been suggested.[79] Further investigations of the molecular size of these polysaccharides would appear to be necessary before any conclusion can be drawn regarding their size in the native state.

TABLE X

The Molecular Weight of Some Pectic Materials (See also Table IX)

Pectic material	*Derivative studied*[a]	*Method*[b]	*Molecular weight*	*Degree of polymerization*	*Frictional ratio* (f/f_o)	*Reference*
Apple pectin	Nitrate	Viscosity	$(30–100)10^3$	165–550	—	71
Apple pectin	Unsubstituted	S. & D.	$(25–35)10^3$	130–190	—	72
Apple pectin (free)	Unsubstituted	S. & D.	$201(10)^3$	1,100	6.9	74
Apple pectin (extracted)	Unsubstituted	S. & D.	$67(10)^3$	370	4.5	74
Apple pectin	Nitrate	Viscosity	$(32–213)10^3$	175–1,160	—	78
Apple pectin	Propionate	Viscosity & O. P.	$(20–150)10^3$	110–820	—	79
Apple pectin	Unsubstituted	O. P.	$140(10)^3$	770	—	81
Apple pectin	Unsubstituted	Viscosity	$(23–71)10^3$	125–390	—	75
Lemon pectin (free)	Unsubstituted	S. & D.	$414(10)^3$	2,250	7.4	74
Lemon pectin (extracted)	Unsubstituted	S. & D.	$89(10)^3$	490	5.1	74
Lemon pectin	Propionate	Viscosity	$(20–150)10^3$	110–820	—	79
Orange pectin (free)	Unsubstituted	S. & D.	$(25–35)10^3$	130–190	—	72
Plum & pear pectin (free)	Unsubstituted	S. & D.	$(40–50)10^3$	220–270	—	72
Flax pectin	Unsubstituted	O. P.	$100(10)^3$	550	—	81
Flax pectin	Unsubstituted	S. & D.	$64(10)^3$	350	4.5	74

[a] Unsubstituted derivatives were measured in aqueous salt solutions.
[b] S. & D. = sedimentation and diffusion measurements; O. P. = osmotic pressure measurements.

b. Alginic Acid.—Alginic acid is associated with the skeletal carbohydrate of the brown-seaweeds (*Phaeophyceae*), and is usually isolated by extracting the acid-treated fronds with 0.5% sodium carbonate solution. Structural studies have shown the polysaccharide to consist of

D-mannuronic acid residues joined by β-1,4-glycosidic linkages,[84] and have a chain length of about 100 hexuronic acid units.[85]

Investigations by X-ray methods have indicated that alginic acid possesses a fiber structure analogous to that of cellulose.[86] An estimate of the molecular weight was obtained by Heen[87] from a study of the viscosity of solutions of the acid in 2 *N* sodium hydroxide. The values found, (14,100–15,400), corresponded to a degree of polymerization of about 80, but these were considered to be approximations only, as it was uncertain whether or not Staudinger's formula could be applied.

Säverborn[74] has carried out a careful study of the physical properties of alginic acid. The polysaccharide in solutions of low ionic strength was found to exhibit an electroviscous effect which was eliminated when higher salt concentrations were used, as was a similar dependence of diffusion and sedimentation constants upon ionic strength. The value of the molecular weight of a laboratory-prepared alginic acid was found to be 50,000 ($S_{20} = 2.4(10)^{-13}: D_{20} = 3.0(10)^{-7}: V = 0.605$), whilst that for a commercial sample was 75,000 ($S_{20} = 3.0(10)^{-13}: D_{20} = 2.5(10$ The values of the frictional ratio ($f/f_0 = 3.1$ and 3.2) indicated that the molecules were elongated in solution. An idea of the polydispersity of the material was obtained by comparing the molecular weight from sedimentation and diffusion measurements with that from sedimentation equilibrium measurements ($S_E = 69{,}000$). Viscosity measurements showed the value of K_m in Staudinger's equation to be $27.0(10)^{-4}$ solutions in 0.2 *N* sodium carbonate.

The molecular weight of the sodium salt of alginic acid has been determined by measurements of osmotic pressure in sodium chloride solution.[88] The Π/C versus C curve was found to be independent of concentration for values between 0.2 *N* and 0.5 *N*, and the slope of the curve was the same for a series of seven fractions with values of molecular weight between 48,000 and 186,000 (220–860 D-mannuronic acid units). Intrinsic viscosity measurements showed the coefficient α in the modified Staudinger's equation[89] to be 1.0 and the approximate value of K_m to be $15(10)^{-4}$.

From the evidence yet available it is not possible to determine definitely whether alginic acid possesses a linear or a slightly branched structure.

(84) E. L. Hirst, J. K. N. Jones and Winifred O. Jones, *J. Chem. Soc.*, 1880 (1

(85) S. K. Chanda, E. L. Hirst, (the late) E. G. V. Percival and A. G. Ross, *Chem. Soc.*, 1833 (1952).

(86) H. Kringstad and G. Lunde, *Kolloid Z.*, **83**, 202 (1938).

(87) E. Heen, *Kolloid Z.*, **83**, 204 (1938).

(88) F. G. Donnan and R. C. Ross, *Canad. J. Res.*, **28B**, 105 (1950).

(89) See equation (6), page 294.

VI. The Molecular Weights of Polysaccharides Containing More Than One Type of Structural Unit

1. *Plant Gums and Mucilages*

There is no clear distinction between these two groups of complex substances, although attempts have been made to classify them.[90]

Plant gums, which occur as exudates on the bark or leaves of trees, are salts of polyuronic acids and are usually freely soluble in water. It is often difficult to decide whether such polysaccharides are homogeneous, but a beginning has now been made in the determination of their structures. Weak linkages have been shown to be present, and it is possible to remove portions of the molecules that are readily hydrolyzable by mild acid, leaving a more stable acidic residue. The structure of the gums appears to depend upon the source, and some studies are complicated by the fact that the botanical origin of the gum was not known with certainty.

The closely related mucilages are widely distributed in intercellular material and in the cell contents, and they possess the same type of molecular structure as the gums. Mucilages may be either neutral or acidic (when uronic acid residues are present). Again, little is known of the finer details of molecular structure and it is often difficult to prove whether or not the material is homogeneous.

Molecular weight determinations on both groups of polysaccharides are not extensive.

a. Gum Arabic.—Gum arabic is exuded from fissures or wounds in the bark of various species of *Acacia*. Structural determinations have shown this complex polysaccharide to be highly branched.

Investigations of the particle size of gum arabic were first carried out by Oakley,[91] using osmotic pressure measurements on aqueous solutions. The molecular weight values were found to depend upon the pre-treatment of the material and the composition of the electrolyte solutions used. This behavior was assumed to be due either to the particles aggregated to different extents under different experimental conditions, or to a varying suppression of the Donnan effect. In a later paper,[92] the Donnan effect was eliminated by the use of solutions at high ionic strengths, and the molecular weights of various salts of the gum were determined. These salts of the gum were prepared by dialysis of the

(90) See J. K. N. Jones and F. Smith, *Advances in Carbohydrate Chem.*, **4**, 243 (1949), and E. L. Hirst, *Research*, **4**, 106 (1951).

(91) H. B. Oakley, *Trans. Faraday Soc.*, **31**, 136 (1935).

(92) H. B. Oakley, *Trans. Faraday Soc.*, **32**, 1360 (1936).

acidic gum against the appropriate chloride. Irregular Π/C vers
curves were obtained. The sodium salt of the gum, when measur
0.1 *N* sodium chloride solution, showed a sudden decrease in the va
Π/C within the concentration range 0.15–0.30%, a similar change
found for the calcium salt at concentrations less than 0.3%. As d
minations of osmotic pressure at these low concentrations were subj
to large errors, Oakley suggested that ultracentrifuge measure
were the only method of investigating this behavior. The mole
weights of the bases used were given as between 191,000 and 24
when extrapolations of Π/C to zero concentration were made, negle
the anomalous behavior at low concentrations.

This behavior was not confirmed by Säverborn[74] when measurin
sedimentation and diffusion constants of gum arabic. Although
constants varied with the electrolyte media at low salt concentrat
this effect was eliminated in solutions of high ionic strength.
apparent molecular weight varied with the acidity of the medium (T
XI), but contrary to the findings of Oakley,[91] no increase in the val
the sedimentation constant at low concentrations was found. The
particles were elongated in solution ($f/f_0 = 2.2$), a result that was
firmed by streaming birefringence measurements. Sedimentation e
librium measurements showed the molecular weight of the sodium sa
be 400,000, and it was suggested that the large difference between the
values for the molecular weight was due to aggregation. The view
the pre-treatment, or the change of media, altered the aggregation fo
was confirmed by the large variation in polydispersity of two sampl

TABLE XI
Molecular Constants for Gum Arabic[74]

Polysaccharide	*Solvent*	pH	$D_{20}(10)^7$	$S_{20}(10)^{13}$	*Frictional ratio* (f/f_0)	*Molec weig*
Acid gum arabic	0.2 *N* NaCl	7.0	2.3	10.7	2.2	298,
Acid gum arabic	0.2 *N* NaCl	1.1	2.2	11.5	2.3	326,
Sodium salt	0.2 *N* NaCl	7.0	2.6	10.4	2.1	256,

b. Gum Tragacanth.—Various species of shrubs belonging to the g
Astragalus (of the order *Leguminosae*) exude this gum. It has
shown by Smith[90] to be a mixture of the salt of a complex polyuronic
and a neutral polysaccharide, mainly an araban.

Gralén and Kärrholm have investigated the behavior of a pur
tragathanth gum in the ultracentrifuge.[93] Sedimentation and diff

(93) N. Gralén and Marianne Kärrholm, *J. Colloid Sci.*, **5**, 21 (1950).

measurements carried out in 0.2 *N* sodium chloride solution indicated that the molecular weight was 840,000 ($S_{20} = 8.0(10)^{-13}$:$D_{20} = 0.59(10)^{-7}$:V = 0.607), this value being only approximate as 30–40% of the material consisted of higher molecular weight aggregates which immediately sedimented to the bottom of the cell. The soluble portion of the material was polydisperse.

The auto-hydrolytic effect of boiling an aqueous solution was also investigated by following changes in the sedimentation and diffusion constants and the intrinsic viscosity. Although the measurements were complicated by the fact that the insoluble portion dissolved as boiling proceeded, the results indicated that the gum molecules were long chains with small adjacent side chains, and that hydrolysis attacked the weaker side chains.

c. Karaya Gum.—Karaya gum is the dried exudate from the stem of *Sterculia urens*, a native tree of India. Very little is known of the composition of this gum.[94] Kubal and Gralén[95] have found that the molecular weight from sedimentation and diffusion measurements in 0.2 *N* sodium chloride solution was 9,500,000 ($S_{20} = 17.5(10)^{-13}$:$D_{20} = 0.14(10)^{-7}$:V = 0.68). Both the sedimentation and the diffusion constants were strongly dependent on the concentration and the material was fairly homogeneous. The particles were not spherical in solution since f/f_0 was 2.4.

d. Locust Bean Mucilage.—This polysaccharide is the dried aqueous extract of the seeds of the locust bean tree (*Ceratonia Siliqua*). It contains D-mannose and D-galactose in the molecular proportion of 1:3 or 1:4, and may be a mixture of polysaccharides.[96]

The material has been studied by Gralén and Kubal in the ultracentrifuge.[95] These measurements indicated that the molecular weight was 310,000 ($S_{20} = (3.6)10^{-13}$:$D_{20} = (0.85)10^{-7}$:V = 0.66), the high dependence of the sedimentation constant on concentration probably being accounted for by the high intrinsic viscosity. The mucilage particles were not very asymmetric ($f/f_0 = 1.24$) and the material was found to be fairly homogeneous.

e. Arabogalactans.—The most studied arabogalactan is that obtained from the ϵ-galactan of larch wood. This water-soluble polysaccharide contains L-arabinose and D-galactose in a 1:6 molecular proportion, but on chemical evidence it was not known whether this substance was a co-polymer or a mixture of polysaccharides, one of which was a

(94) C. L. Mantell, "The Water Soluble Gums," Rheinhold Pub. Corp., New York (1947).

(95) J. V. Kubal and N. Gralén, *J. Colloid Sci.*, **3**, 457 (1948).

(96) B. W. Lew, and R. A. Gortner, *Arch. Biochem.*, **1**, 325 (1943).

galactan.[97] However, Svedberg and Mosiman,[61] studying the mat in the ultracentrifuge, obtained sedimentation diagrams showing presence of two components, a galactan and an arabogalactan. molecular weight of the arabogalactan was 16,000.

TABLE XII

The Molecular Weight of Some Plant Gums and Mucilages

Polysaccharide[a]	*Method*	*Frictional ratio* (f/f_0)	*Molecular weight*	*Structure and shape*
Gum Arabic	Osmotic pressure	—	191,000–245,000	A highly branched molecule possessing an elongated shape in solution
	Sedimentation and Diffusion	2.2	256,000–326,000	
Gum Tragacanth	Sedimentation and Diffusion	—	840,000	Preceding description applies
Karaya gum	Sedimentation and Diffusion	2.4	9,500,000	Preceding description applies
Locust Bean mucilage	Sedimentation and Diffusion	1.24	310,000	Molecule not very asymmetric
Arabo-galactan from larch wood	Sedimentation and Diffusion	—	16,000	A highly branched molecule

[a] All measurements were carried out using the unsubstituted polysaccharides in aqueous salt solu

The existence of the mixture of polysaccharides was confirmed Hirst and co-workers,[63] who obtained two fractions from the methyla ϵ-galactan, one containing only a trace of pentose, whilst the other rich in pentosan. The structure of the arabogalactan portion which investigated was found to be highly branched.

Molecular weight measurements on the fractionated material necessary, although Husemann[29] has measured the molecular weight of

(97) F. C. Peterson, A. J. Barry, H. Umkauf and L. E. Wise, *J. Am. Chem. S* **62,** 2361 (1940); E. V. White, *ibid.*, **63,** 2871 (1941); **64,** 302, 1507 (1942).

mixture by osmotic pressure. These measurements showed that the degree of polymerization was about 220 (this value being unaltered on esterification), and the relationship between molecular weight and intrinsic viscosity suggested that the material was highly branched.

2. *Mucopolysaccharides*

Mucopolysaccharides are generally found to have a small but significant proportion of associated protein material. The results of structural determinations in this field have been reviewed recently.[98] In most instances, rather drastic methods, including the use of alkali, are needed to remove the contaminating protein and so obtain a soluble product. Degradation may possibly accompany such isolation procedures, and dissociation of the protein–polysaccharide complex may also completely alter the physical properties of the product.

As comparatively little is known of the detailed structure of most of the complex polysaccharides in this extensive group, relatively few physical measurements have been made.

a. Microbiological Polysaccharides.—All such polysaccharides, whether synthesized exocellularly or somatically, may occur in combination with protein or amino-acid residues, and the removal of this material is often difficult. The microbiological polysaccharides that will now be discussed in order are those from *Penicillium* species, from *Eberthella typhosa*, from *Mycobacterium tuberculosis* and from *Pneumococcus*.

Penicillium Charlesii G. Smith. This mould produces two low molecular weight polysaccharides, mannocarolose.[99] (a mannan) and galactocarolose[100] (a galactan). Mannocarolose was shown by methylation and hydrolysis to possess a chain-length of 8 hexose residues, a value confirmed by the estimation of iodine numbers on the unsubstituted polysaccharide. The mannocarolose molecule was shown to be unbranched when the molecular weight of its methylated derivative was found by Rast's method to be 1622 (about 8 hexose units). Similar methods showed the galactocarolose to possess an unbranched structure with an average chain-length of 9–10 D-galactose units.

Penicillium luteum Zukal. A neutral polysaccharide, luteose, is produced by the mild alkaline hydrolysis of the malonyl residues from luteic acid, a metabolic product of this mould. Luteose was shown to be a poly-D-glucose in which the hexose units were joined by β-1,6-glycosidic

(98) M. Stacey, *Advances in Carbohydrate Chem.*, **2**, 161 (1946).
(99) W. N. Haworth, H. Raistrick and M. Stacey, *Biochem. J.*, **29**, 612 (1935).
(100) W. N. Haworth, H. Raistrick and M. Stacey, *Biochem J.*, **31**, 640 (1937).

linkages.[101] It was suggested that the molecular structure might be the closed chain type, for although no end-group was found in methylation studies, the molecular weight of the methylated derivati determined by osmotic pressure measurements, was 17,000 (84 hex units). Recently two new polysaccharides have been isolated by fr tionating the dialyzed culture medium of this mould,[102] a dextrorotat neutral polysaccharide A containing 88% D-galactose, and a levorotat acidic polysaccharide B containing 64% D-glucose. Sedimentation diffusion measurements on these products have been carried out Ogston.[103] Both polysaccharides were polydisperse, A consisting o homogeneous and a heterogeneous fraction, and B being complet heterogeneous. The values of the molecular weights obtained were a approximate, as for both polysaccharides a large proportion of material was not refracting (Table XIII).

TABLE XIII

Molecular Constants for Penicillium Luteum Zukal *Polysaccharides*[103]
(Partial molar volume (V) = 0.62. Solvent: Phosphate buffer)

Fraction	*Per cent material refracting*	$S_{20}(10)^{13}$	$D_{20(corr.)}(10)^{7}$	*Molecular weight*
Polysaccharide A (dextrorotatory)				
(i) Homogeneous[a]	0.84	4.13	5.5	50,000
(ii) Heterogeneous[b]	0.84	*ca.* 5.2	1.9	170,000
Polysaccharide B (levorotatory)				
Heterogeneous	0.64	1.79	8.32	15,000

[a] This fraction represented 43 % of the dextrorotatory polysaccharide A.
[b] This fraction represented 57 % of the dextrorotatory polysaccharide B.

Eberthella typhosa. The polysaccharide obtained by the hydrol of the complex bacterial antigen, isolated from the bacterial cells tryptic digestion,[104] has been examined in the ultracentrifuge by Philpot.[105] Diffusion measurements were not carried out, but assuming that the mo cule was spherical, the sedimentation constant ($S_{20} = 1.2(10)^{-13}$) in cated a molecular weight of about 10,000 (60 hexose units). The po saccharide was found to be relatively homogeneous in these measureme

(101) C. G. Anderson, W. N. Haworth, H. Raistrick and M. Stacey, *Biochem.* **33,** 272 (1939).
(102) G. C. Freeman and Catherine S. Macpherson, *Biochem. J.*, **45,** 179 (19
(103) A. G. Ogston, *Biochem. J.*, **45,** 189 (1949).
(104) G. G. Freeman, *Biochem. J.*, **36,** 340 (1942).
(105) J. St. L. Philpot, *Biochem. J.*, **36,** 355 (1942).

Mycobacterium tuberculosis. Many polysaccharides from this organism have been studied[106] and have been isolated from the culture medium, the somatic portion of the cell, and the cell lipoids. Only in a few cases have the structures been definitely established.

The polysaccharides associated with the culture media from different strains of *Mycobacterium tuberculosis* have been examined in the ultracentrifuge. Seibert[107] separated a seriologically active polysaccharide from human-type bacilli. It was homogeneous and had a molecular weight of 9,000 ($S_{20} = 1.6(10)^{-13}$:$D_{20} = 11(10)^{7}$:V = 0.619:frictional ratio (f/f_0) = 1.5). Similar results were obtained by Tennent and Watson,[108] who found a molecular weight of 7,300 and a frictional ratio of 1.4 for the polysaccharide from this strain. Measurements on the polysaccharides from avaian and *Mycobacterium phlei* strains showed that these had very similar molecular characteristics, although that associated with the leprosy bacilli was much smaller, having a molecular weight of 2500, and spherical. The sedimentation constant was in all cases found to be independent of concentration (Table XIV).

TABLE XIV

Sedimentation and Diffusion Data for Some Mycobacterium tuberculosis *Polysaccharides*[108]

(*Partial molar volume* (*V*) = *0.619. Solvent: Phosphate buffer* (pH *7.4*))

Strain	$S_{20}(10)^{13}$	$D_{20}(10)^{7}$	*Frictional ratio* (f/f_0)	*Molecular weight*
Human	1.39	12.4	1.40	7,200
Avian	1.54	13.6	—	7,300
Mycobacterium phlei	1.63	14.7	—	7,200
Leprosy	0.97	24.9	0.99	2,500

The Pneumococcal Polysaccharides. Specific polysaccharides from Types I, II and III *Pneumococcus,* which had been obtained in relatively undegraded forms, have been examined by Stacey and Record.[109] In contrast to the results of Tennent and Watson, both the sedimentation and the diffusion constants were found to be very dependent on the concentration. The polysaccharides were polydisperse, with molecular weights in the range $(10)^5$–$(10)^6$, and they possessed an elongated form in solution (Table XV).

(106) M. Stacey and P. W. Kent, *Advances in Carbohydrate Chem.*, **3,** 311 (1948).

(107) Florence B. Seibert, K. O. Pederson and A. Tiselius, *J. Exptl. Med.*, **68,** 413 (1938).

(108) D. M. Tennent and D. W. Watson, *J. Immunol.*, **45,** 179 (1942).

(109) B. R. Record and M. Stacey, *J. Chem. Soc.*, 1561 (1948).

TABLE XV

Sedimentation and Diffusion Data for Types I, II and III Pneumococcus[108] (*Partial molar volume (V) = 0.537. Solvent: 0.20 N NaCl*)

Type	$S_{20}(10)^{13}$	$D_{20}(10)^{7}$	*Frictional ratio* (f/f_0)	*Molecular weight*
I	6.5	2.00	3.2	171,000
II	7.2	0.75	6.0	540,000
III	4.3	1.60	4.3	141,000

It is convenient to mention here a high molecular weight glyc isolated by Chargaff and Moore[110] from avian tubercle bacilli by speed sedimentation of a borate buffer extract of the cells and extrac of the polysaccharide by trichloroacetic acid. Some of the fract obtained were monodisperse in the ultracentrifuge, having a molec weight of $12(10)^6$ ($S_{20} = 170(10)^{-13}$: $D_{20} = 0.92(10)^{-7}$: V = 0.63). frictional ratio ($f/f_0 = 1.59$) showed that the glycogen molecule posse an elongated form, and the high value of the molecular weight was firmed by calculations, using the viscosity increment and partial spe volume, as $13.2(10)^6$.

The molecular weight of the methylated polysaccharide used in st tural determinations of a complex specific somatic polysaccharide been determined by osmotic pressure measurements in chloroform s tion as being 12,000.[111]

The molecular weights of the polysaccharides associated with *m bacterium tuberculosis* appear to vary considerably, depending on source and method of extraction.

b. Chondroitin Sulfate.—The calcium salt of this complex poly charide occurs with collagen in animal cartilage, and the extract usually involves saponification. The detailed structure has not yet b definitely established.[112]

The character of the polysaccharide product depends on the extr tion procedure, and it is necessary to study the degradation involv Meyer, using low temperature extraction, obtained a product having intrinsic viscosity of 0.80, which he considered undegraded in compari with that of Fürth and Bruno,[113] who found $[\eta] = 0.50$ with a cor sponding molecular weight of 17,200. The presence of an end-group

(110) E. Chargaff and D. H. Moore, *J. Biol. Chem.*, **155,** 493 (1944).

(111) Sir Norman Haworth, P. W. Kent and M. Stacey, *J. Chem. Soc.*, 1211 (19

(112) K. H. Meyer, M. E. Odier and A. E. Seigrist, *Helv. Chim. Acta*, **31,** 1 (1948).

(113) O. Fürth and T. Bruno, *Biochem. Z.*, **294,** 153 (1937).

the molecule was shown by iodine titration, a molecular weight of about 30,000 being indicated by this method. Also from viscosity measurements Meyer concluded that the molecule possessed an unbranched structure. Recently Blix and Snellman[114] have extracted the polysaccharide using only calcium chloride solutions. The product had a molecular weight of 260,000 from viscosity and streaming birefringence measurements, and in the presence of alkali it broke down to a stable unit with a molecular weight of 30,000–40,000. Unfortunately, it was not possible to decide whether this behavior was due to degradation or disaggregation of micelles.

It is difficult to correlate the results of the physical and chemical investigations on this polysaccharide, and further investigations appear to be necessary. A degraded product having a molecular weight of about 30,000 appears to be easily formed.

c. Blood-Group Polysaccharides.—Structural studies on these complex glyco-polypeptides have been reviewed recently by Bray and Stacey.[115] Little would appear to be known of the detailed structure of the molecules.

The first physico-chemical determination appears to be that of Kekwick.[116] He examined in the ultracentrifuge the material prepared from ovarian cyst fluid which showed blood-group A specificity.[117] This product was electrophoretically homogeneous and only one component was apparent in the sedimentation measurements. The complex was moderately polydisperse and possessed a highly asymmetric shape ($f/f_0 = 3.2$). The values of the sedimentation and diffusion constants, interpolated graphically at a given concentration, corresponded to a mean molecular weight of 260,000

$$(S_{20} = (5.4\text{–}6.7)10^{-13} : D_{20} = (1.60\text{–}1.70)10^{-7} : V = 0.635).$$

A similar product isolated from hog gastric mucin has been studied by Pardee and Blaker.[117a] Light-scattering data indicated a molecular weight of $(9.0\text{–}1.1)10^5$, and a similar value was obtained from viscosity and diffraction measurements ($D_{20} = (1.03\text{–}1.12)10^{-7}$). Osmotic pressure measurements however gave a value of 220,000, and the discrepancy between the two figures was taken as indicating the sample was inhomogeneous. The viscosity measurements also indicated the complex was highly asymmetrical.

(114) G. Blix and O. Snellman, *Arkiv Kemi Mineral. Geol.*, **A19**, No. 32 (1945).
(115) H. G. Bray and M. Stacey, *Advances in Carbohydrate Chem.*, **4**, 37 (1949).
(116) R. A. Kekwick, *Biochem. J.*, **46**, 438 (1950).
(117) D. Aminoff, W. T. J. Morgan and Winifred M. Watkins, *Biochem. J.*, **46**, 426 (1950).
(117a) A. B. Pardee and R. H. Blaker, *Proc. Soc. Exp. Biol. Med.*, **78**, 589 (1951).

d. Heparin.—Heparin, the blood-anticoagulant polysaccharid pears to be firmly bound with protein material in tissue. The str of this polysaccharide is not definitely known although several fo have been suggested.[118,119] It contains D-glucuronic and D-gluco residues together with a large number of sulfuric ester groupings. determinations of molecular weight have been made. Meyer quoted a molecular weight of 15,000, and Wolfrom and co-worke figure of 20,000 from diffusion measurements, no experimental being given in either case.

e. Hyaluronic Acid.—This polysaccharide has been isolated sources including vitreous humour, synovial fluid, and umbilica where it occurs as a complex with protein. Structural determin are as yet incomplete.[120–124] There is evidence that degradation accompanies isolation and purification of the product, and that di tion of the polysaccharide–protein complex has a profound influe physical properties. The relative viscosity has been used to follo degradation accompanying different isolation procedures, althou intrinsic viscosity would appear to be a more satisfactory cri Results for different procedures have been summarized;[125] they values for the relative viscosity of between 1.3 and 4.5 (concent 1 g. per liter in various buffered solutions). Although a direct co son of relative viscosity is difficult, Hadidan and Pirie obtained their own method of isolation a product having a relative viscosity in phosphate buffer, which they presumed was less degraded than products.

The electroviscous effect can influence the results of viscosity minations, but this can be avoided by using a sufficiently high con tion of salt[126] when linear specific viscosity concentration versus c tration curves are obtained. Gelation effects with Fe^{++}, Fe^{+++} an ions also occur[127] and so should be avoided.

Solutions of hyaluronic acid exhibit double refraction of flow.

(118) M. L. Wolfrom, R. Montgomery, J. V. Karabinos and P. Rathgeb *Chem. Soc.*, **72,** 5796 (1950).

(119) K. H. Meyer, "Natural and Synthetic High Polymers," 2nd ed. (1950), Interscience Publishers, New York.

(120) K. H. Meyer and J. Fellig, *Experientia,* **6,** 186 (1950).

(121) R. Jeanloz, *Experientia,* **6,** 52 (1950).

(122) M. A. G. Kaye and M. Stacey, *Biochem. J.*, **48,** 249 (1951).

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(127) C. E. Jensen, *Acta Chem. Scand.*, **3,** 584 (1949).

has been measured by Blix and Snellman[128] and used to calculate particle lengths and molecular weights. The length of the hyaluronic acid particle from vitreous humour was estimated as 4800 Å, that from synovial fluid as 7000 Å, and that from umbilical cord as 10,000 Å. Molecular weights of between 200,000 and 400,000 were suggested for these molecules.

The most complete physico-chemical study of hyaluronic acid appears to be that of Ogston and Stanier,[129] who used the hyaluronic acid–protein complex from ox synovial fluid. Degradation of the complex was avoided by isolation using ultrafiltration. A product having a very high relative viscosity was obtained (*i.e.*, $\eta_{rel} = 39$ at a concentration of 1 g. per 1000 ml. in 0.2 *N* NaCl). This would appear to be the most satisfactory method of isolation, as other methods were shown to cause significant degradation, and, in particular, the removal of the protein which formed 30% of the complex) caused significant changes in physico-chemical properties. The molecular size of the complex from sedimentation and diffusion measurements was 8,000,000:

$$(S_{20} = 13(10)^{-13}: D_{20} = 1.0(10)^{-7}: V = 0.69),$$

and the molecule was found to possess an elongated shape ($f/f_0 = 3.25$). The complex sedimented as a single component. Difficulties in the extrapolation of D_{20} to zero concentration were presumed to be due to experimental error, and the values of D_{20} at higher concentrations were used to calculate the molecular weight. Viscosity measurements at low rates of shear indicated that there was considerable interaction between these particles, even at low concentrations. In a later paper Ogston and Stanier[130] have described further viscosity and streaming birefringence measurements on the same polymer solution, and modified their previous deductions. They claimed that these new results showed the molecular weight of the complex to be $8(10)^6$. Methods were deduced for calculating the variation of diffusion constant with concentration, and the corrected value was found to be $D_{20} = 0.065(10)^{-7}$. Several methods of calculating the axial ratio showed that this was small (not greater than 3), and the molecule was regarded as being a flexible chain which, being highly solvated, approximated a spherical shape.

In comparison, the results of periodate oxidation and the determination of iodine numbers, using sodium hyaluronate, by Jeanloz[121] would indicate that the polysaccharide was linear, and had a molecular weight of 18–24,000 (50 units). A further decrease in size of the molecule which

(128) G. Blix and O. Snellman, *Nature*, **153**, 587 (1944).
(129) A. G. Ogston and J. E. Stanier, *Biochem. J.*, **46**, 364 (1950).
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TABLE XVI

The Molecular Weight of Some Mucopolysaccharides

Polysaccharide	*Derivative studied*	*Method*[a]	*Solvent*	*Frictional ratio,* (f/f_0)	*Molecular weight*	*D. P.*	*Molecular structure*	*Ref.*
Microbiological, produced by *P. Charlesii* G. Smith								
(A) Mannocarolose	Methyl ether	Rast	—	—	1,750	8–9	Unbranched linear	99
(B) Galactocarolose	Methyl ether	Rast	—	—	1,950	9–10	Unbranched linear	100
Microbiological, produced by *P. luteum* Zukal								
Luteose	Methyl ether	O. P.	Chloroform	—	17,000	84	Closed chain type	101
Dextro-polysaccharide	Unsubstituted	S. & D.	Phosphate buffer	—	i) $50(10)^3$	*ca.* 300	Not known	103
					ii) $170(10)^3$	*ca.* 950	Not known	103
Levo-polysaccharide	Unsubstituted	S. & D.	Phosphate buffer	—	15,000	*ca.* 80	Not known	103
Microbiological, produced by *Eberthella typhosa*	Unsubstituted	S.	0.2 *N* NaCl	—	10,000	*ca.* 60	Not known	105
Microbiological, produced by *M. tuberculosis*								
Human strain	Unsubstituted	S. & D.	Phosphate buffer	1.5	9,000	—	All probably highly branched molecules possessing	107
Human strain	Unsubstituted	S. & D.	Phosphate	1.4	7,300	—		108
Human strain (somatic)	Methyl ether	O. P.	Chloroform	—	12,000	*ca.* 60	an elongated shape	111
	Unsubstituted	S. & D.	Phosphate	—	7,200	—	in solution	108

	Unsubstituted	S. & D.	Phosphate buffer	1.4	7,300	—	cules possessing	108
Human strain (somatic)	Methyl ether	O. P.	Chloroform	—	12,000	*ca.* 60	an elongated shape	111
M. phlei	Unsubstituted	S. & D.	Phosphate buffer	—	7,200	—	in solution	108
Avian	Unsubstituted	S. & D.	Phosphate	—	7,300	—		108
Avian (glycogen)	Unsubstituted	S. & D.	buffer	1.59	12(10)[6]	*ca.* 65,000		110
Microbiological, produced by Leprosy bacilli	Unsubstituted	S. & D.	Phosphate buffer	0.99	2,500	—	Spherical	108
Microbiological, produced by:								
Type I *Pneumococcus*	Unsubstituted	S. & D.	NaCl sol.	3.2	171,000	*ca.* 800	Molecules possess-	109
Type II *Pneumococcus*	Unsubstituted	S. & D.	NaCl sol.	6.0	540,000	—	ing elongated	109
Type III *Pneumococcus*	Unsubstituted	S. & D.	NaCl sol.	4.3	141,000	*ca.* 700	shape in solution	109
Chondroitin sulfate	Unsubstituted	Iodine number	—	—	31,600	—	Unbranched linear	112
	Unsubstituted	Birefringence		—	260,000	—	Elongated	114
Blood group polysaccharides	Unsubstituted	S. & D.	Phosphate buffer	3.2	260,000	—	Highly branched molecule elon-	116
	Unsubstituted	D. and τ	Buffer	—	(9.0–1.1)10[6]	—	gated in solution	117(a)
Heparin	Unsubstituted	O. P.	?	—	15,000	—	?	118
	Unsubstituted	D.	?	—	20,000	—	?	119
Hyaluronic acid	Unsubstituted	S. & D.	Buffer	3	8(10)[6]	*ca.* 20,000	Flexible chain molecule	130
	Unsubstituted	Birefringence	?	—	(2–4)10[5]	*ca.* 800	Elongated in solution	128
	Unsubstituted	Iodine number	—	—	(18–24)10[3]	50	Unbranched linear	121

[a] O.P. = osmotic pressure measurements: S. & D. = sedimentation and diffusion measurements: τ = light-scattering measurements.

occurred on heating was thought to be due to disaggregation an degradation. Jeanloz emphasized that the value of size obtained these conditions did not necessarily bear any relation to that from cal measurements. The linear nature of the polysaccharide wa inferred by Blix[124] from periodate oxidation studies.

Further investigations of the physical properties of hyaluroni are clearly necessary. The method of extraction is important an cedures which do not involve degradation should be used. There possibility also that protein in the polysaccharide-complex may purely physical aggregation and correspondingly high molecular we

The result of molecular weight measurements carried out o mucopolysaccharides are summarized in Table XVI.

VII. Conclusions

The results of the measurements of the shape and size of po charides show that few such polymers have been adequately c terized. Relatively little is known of their size in the native stat the problems concerned with the isolation of these substances, an preparation of derivatives have not been completely solved. A mount problem is the avoidance of degradation. Nevertheless, va information on the high-polymer characteristics of a number of d polysaccharide materials has been obtained. Many polysacchari known chemical structure have not yet been examined, and the field is an attractive one for further investigations.

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APPARATUS AND TECHNIQUE

MPLE ELECTROMETER FOR SMALL-SCALE POTENTIOMETRIC TITRATIONS

By D. M. W. Anderson and C. T. Greenwood

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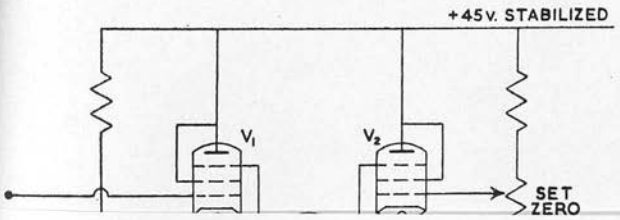
n electrometer was required as the null-point indicator in measurement of the uptake of iodine by starch and other ysaccharides using the differential potentiometric titration hnique of Gilbert and Marriott.[1] The investigation of nples on a small scale necessitated an instrument of high sitivity; at the same time a simple design was aimed at so t determinations could be made on a routine basis.

Many electrometer circuits, designed to achieve sensitivity h minimum zero-drift and instability, have been described.[2] mplete elimination of zero-drift has been claimed,[3] but this generally been attained by use of internal compensation,[4] l-balance,[5] the continuous charging of batteries,[6] or negative d-back and stages of D.C. amplification.[7] Consequently, se circuits require some form of tuning, and are not as simple was desired. In most instances, elaborate precautions with gard to external shielding, thermostatting and earthing were o found to be necessary.[8]

Preliminary investigations were made using an electrometer ode (grid current 10^{-15} amp.) as one of the arms in a Wheat- ne bridge circuit built into an air-tight vibration-free box, m which light was excluded. Using a spot galvanometer of nsitivity 109 mm. per micro-amp. and internal resistance 2 ohms, the sensitivity obtained was 6 mm. per mv. Zero ft was reduced to an average of 0·5 mm. per minute by using od quality components, allowing adequate wattage dissipation all resistors, and using aged and well-charged accumulators. zero drift of 1—2 mm. per minute has been commonly claimed r circuits of similar sensitivity).[8, 9] Neither the sensitivity r the rate of zero drift obtained was considered to meet the ndards required. In addition, the system was so susceptible external interference despite the instalment of a virtual earth stem, the provision of an equipotential shield,[10] and careful reening, that it was rejected.

The circuit finally adopted employs the well-known principle using a matched pair of valves as two of the resistances in a Wheatstone network,[11] with the other two bridge resistors in this se acting as cathode loads for the valves, as shown in Fig. 1.



that given by an electrometer triode, no undesirable effects in solution have been observed.)

The valves (Type VR116) are a matched pair, selected from ten which were available. This type was chosen because of its sturdy filament construction and good heat inertia, which enabled the grid current to be reduced as far as possible by under-running the valves as follows: (*a*) The nominal 6·3 v. filaments are run off 4 volts, supplied by three parallel pairs of 2-volt accumulators, aged and well-charged;[12] (*b*) H.T. voltage is + 45 v., obtained from an electronically stabilized power unit by means of a potential divider chain.

The only shielding employed is conventional screening of the input coaxial cable to the grid of V.1. The metallized envelope of the valves is earthed, but screened-type grid top-caps are not required, despite the presence in the same room of motors and other equipment capable of electrical interference. It has not even been found necessary to shield the valves from light, or from local changes in room temperature.

The constancy of zero attained is shown by the following typical figures obtained in different experiments:

Table I

Time in mins.	..	0	3	6	9	12	18
Galvo. defln. (mm.)	..	0	0	0	0	+1	+1
Time in mins.	..	0	10	30	50	70	100
Galvo. defln. (mm.)	..	0	+0·5	+0·5	0	+0·5	+2·5

Response is perfectly linear over the range 0—5 mv. input, obtained from a Tinsley potentiometer. The sensitivity (using the galvanometer mentioned above) is 28 mm. per mv., which in conjunction with the constancy of zero, enables potentials of 0·01 mv. to be measured.

With this sensitivity, an uptake of 2×10^{-10} moles iodine per mg. polysaccharide can be detected. This has enabled the iodine uptake of lichenin, Floridean starch, amylodextrin and various glycogen samples to be studied, in addition to routine determinations on starch and its components. Sample weights of the order of 2 mg. of amylose, 12 mg. of a starch, and 30 mg. of highly branched polysaccharides are sufficient for characterization. It is hoped to publish the results of this work elsewhere.

In its present form, this circuit is applicable to many potentiometric titrations, having the advantages of inexpensive and simple construction, high sensitivity and accuracy, freedom from external interference, and simplicity in manipulation. While sensitivity can be increased by incorporating a more sensitive galvanometer, this can be achieved, and the use of the circuit extended to measurements with glass electrodes, by using valves of higher mutual conductance.

Acknowledgment

The authors thank Prof. E. L. Hirst, F.R.S., for his interest in this work, and Mr. C. H. C. Matthews for invaluable dis-

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CHARACTERIZATION OF BRANCHED α : 1-4-GLUCOSANS

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uring recent potentiometric studies of the iodine-uptake any branched α : 1-4-glucosans,[1] it has been observed that difference between the binding-power of normal glycogens amylopectins is sufficiently large to characterize the two cture-types.

g. 1 shows typical curves obtained: the slopes of the es for the amylopectins from barley,[2] *Hevea brasiliensis* [3] and oat[4] starches (curves 1, 2 and 3, respectively) are e distinct from those of *Ascaris lumbricoides* and rabbit liver ogens (curves 4 and 5). In all samples so far examined, the nal glycogen-type structure shows no evidence of any ne binding-power due to linear material in the early stages he titration (i.e. for total I_2-concentration $< 1 \times 10^{-6}$M). preferential iodine-uptake by traces of such material rring in all the amylopectin-type curves has been corrected by extrapolating the curve for the branched material to zero l iodine concentration, and hence only uptake by branched ecules is being compared.

n this basis, waxy maize starch behaves as a typical amylo-in (curve 6), and the polysaccharide from the ciliate *Tetra-ena pyriformis*[5] as a glycogen (curve 7).

his potentiometric method gives unambiguous characteri-on, and has the advantage of accuracy and reproducibility er standard conditions ([I′] = 0·01M; pH = 5·85; tem-ature = 20° C.); the titration curve can be repeated on the e 10—20 mg. sample; and the polysaccharide can be vered if desired.

n the range of concentrations employed, the amount of ne bound is directly proportional to the free iodine concen-ion. It is possible that the length of unit-chain alone deter-es the amount bound by any branched glucosan, molecular being of secondary importance, but further investigations abnormal "-type branched glucosans are necessary.

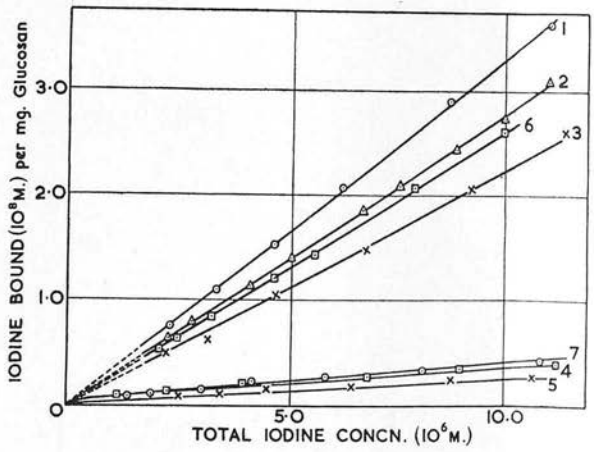

FIG. 1

Comparison of titration curves for branched glucosans

Curve 1. Barley amylopectin (C.L.* = 24—26)
2. *Hevea brasiliensis* seed amylopectin (C.L. = 23)
3. Oat amylopectin
4. *Ascaris lumbricoides* glycogen (C.L. = 12)
5. Rabbit liver glycogen (C.L. = 12)
6. Waxy Maize starch (C.L. = 18—20)
7. *Tetrahymena pyriformis* polysaccharide (C.L. = 12)

* C.L. is the chain-length expressed as the number of glucose units

The authors thank Prof. E. L. Hirst, F.R.S., for his interest in this work, and Dr. D. J. Manners for providing the samples of glycogen investigated.

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A Physico-Chemical Examination of the Capsular Polysaccharide from an Amylolytic Sheep Rumen Streptococcus

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(*Received* 28 *October* 1953)

The capsular polysaccharide from the amylolytic rumen streptococcus BSO 7 described in the preceding paper has been examined in the ultracentrifuge, and in addition its partial specific volume and limiting viscosity number* have been determined.

METHODS

Solution. The dried polysaccharide was dissolved in a buffer containing 0·2 M-NaCl, 0·0633 M-Na_2HPO_4 and 0·0033 M-KH_2PO_4 (pH 7·8). A 1% (w/v) solution was diluted by weight as required and used for all measurements. (Weights were corrected for 7·2% of ash present in the material.)

Sedimentation. Rates of sedimentation were determined using an electrically driven 'Spinco' ultracentrifuge (Specialized Instruments Corporation, Belmont, California, U.S.A.) at rotor speeds of 1000 rev./sec. The pressure in the rotor chamber was less than 1 μ. Hg, and the temperature rise in the rotor itself was about 0·6°/hr. (the refrigerating system was not utilized). Movement of the boundary was followed using a modified Philpot-Svensson optical system. The magnification of this lens system was 2·22, constant over the whole field, and the average distance of the reference line from the axis of rotation was taken as 5·730 cm. (at 1000 rev./sec.). Measurement of the rates of sedimentation were made at several concentrations, the distance through which the boundary had moved being measured, to 0·02 mm., directly from the photographic plate with a travelling microscope. Sedimentation constants were evaluated from the slope of $d \log_{10} x/dt = S/2{\cdot}303$, x being measured in cm. from the centre of rotation and t in seconds from an arbitrary origin, and converted to water at 20°.

Viscosity. An Ostwald type viscometer was used, the essential dimensions being as follows: diameter of capillary, 0·5 mm.; length of capillary, 12·5 cm.; working volume, 2 ml.; mean pressure head, 13·0 cm.; efflux time for solvent, 83·8 sec.; temp. 22·5°. The viscosity number (η_{sp}/c) was determined at several concentrations (where $\eta_{sp} = \eta_{rel} - 1$, and η_{rel} = solution/solvent viscosity ratio), and the limiting

* The nomenclature used in this paper is in accordance with the 'Report on Nomenclature in the Field of Macromolecules of the International Union of Pure and Applied Chemistry', reported in *J. Polym. Sci.* (1952), **8**, 257.

viscosity number $[\eta]$ obtained from the graph of η_{sp}/c against c, as

$$[\eta] = \lim_{c \to 0} (\eta_{sp}/c).$$

Partial specific volume. The densities of solutions of several concentrations were determined using a pyknometer, and the partial specific volume ($\bar{v}$) calculated from the equation (Svedberg & Pedersen, 1940)

$$(1 - \bar{v}\rho) = (1 - w/m)\, dm/dw,$$

where ρ is the density of a solution for which the weight fraction of solute is w and the mass of a given volume m; dm/dw was determined from the linear graph of m against w.

RESULTS

In 0·2 M sodium chloride buffered with phosphate at pH 7·8 the presence of only one component in the polysaccharide was apparent from the sedimentation determinations. The boundaries appeared symmetrical and, for higher concentrations, sharp. Typical sedimentation diagrams are shown in Fig. 1. The values of the sedimentation constants (S_{20}) for three concentrations are recorded in Table 1. Owing to lack of material it was not possible to examine a wider range of concentrations, but a marked dependence of sedimentation constant on concentration was observed. The limiting value of S_{20} was determined using a method suggested by Jullander (1945). In this method S_{20} and $S_{20} \times \eta_{rel}$ are both plotted against c on the same graph and since

$$\lim_{c \to 0} S_{20} = \lim_{c \to 0} S_{20} \times \eta_{rel}$$

the two functions approach the same limiting value and hence define it more exactly. Fig. 2 shows these results and the limiting value of S_{20} is seen to be $3{\cdot}25 \pm 0{\cdot}2 \times 10^{-13}$.

Experimentally determined values of η_{sp}/c are also given in Table 1, together with the extrapolated value of the limiting viscosity number, 3·75. The value for the partial specific volume of the material was found to be 0·60.

The high limiting-viscosity number suggested that the molecules were very asymmetrical, and this figure was used to obtain the approximate axial ratio ($1/d$) and hence the frictional ratio (f/f_0), as a preliminary to the molecular-weight calculation. (This procedure assumes that a value of $f/f_0 > 1$ indicates a deviation from spherical shape when f, the actual molar frictional coefficient $= M(1 - \bar{V}\rho)/S$, and f_0, the molar frictional coefficient of a spherical, non-solvated particle $= 6\pi\eta N\,(3M\bar{V}/4\pi N)^{\frac{1}{3}}$. f/f_0 can then be related to the axial dimensions $1/d$ of the equivalent ellipsoid, where $1/d =$ length (1) of major axis/diameter (d) of equatorial axis.)

The axial ratio ($1/d$) was calculated by the use of Simha's equation (Simha, 1945).

$$\eta_{sp}/\phi = 0{\cdot}233\,(1/d)^{1{\cdot}698},$$

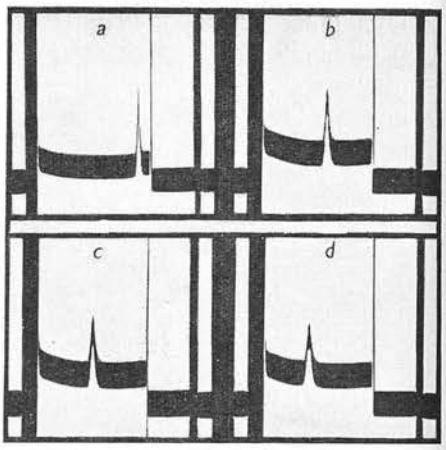

Fig. 1. Sedimentation diagrams for the capsular polysaccharide. Speed, 1000 rev./sec. Solvent, 0·2 M-NaCl buffered with phosphate to pH 7·8. Concentration 0·37 g./100 ml. Times of exposures (*a*) 37 min., (*b*) 129 min., (*c*) 142 min., (*d*) 177 min., from starting the centrifuge.

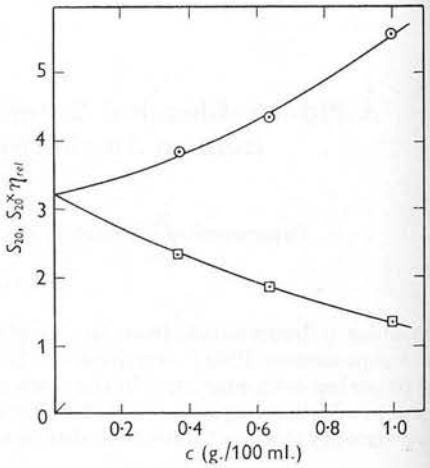

Fig. 2. Determination of limiting value of S_{20} by the method of Jullander (1945). For explanation see the text.

Table 1. *S_{20} and η_{sp}/c values at different values of c for the capsular polysaccharide*

Concentration, c (g./100 ml. solution)	Sedimentation constant $(S_{20}) \times 10^{13}$	Viscosity number (η_{sp}/c)
1·00	1·34	—
0·64	1·87	4·0
0·37	2·34	—
0·34	—	3·9
0·17	—	3·8
0·00	[3·25]*	[3·75]†

* See text and Fig. 2. † By extrapolation.

when $20 \leqslant 1/d \leqslant 100$ and ϕ is the volume fraction of solute. For the concentration units used (g./100 ml. solution) this expression reduces approximately to

$$[\eta]\ 100 = 0{\cdot}233\ (1/d)^{1{\cdot}698},$$

and from this the axial ratio $(1/d)$ was found to be 77. This value represents a frictional ratio (f/f_0) of 3·5, assuming the particles to be prolate spheroids, and using the data of Herzog, Illig & Kudar (1933). (Any hydration effect has been ignored.) The molecular weight M was then calculated from the equation

$$M = 2{\cdot}45 \times 10^{22}\, \overline{V}^{\frac{1}{2}} (f/f_0)^{\frac{3}{2}} [S/(1 - \overline{V}\rho)]^{\frac{3}{2}}.$$

The frictional ratio (f/f_0) of 3·5 gave a value of about 90000. If the molecule was assumed to be spherical and unsolvated (i.e. $f/f_0 = 1$), the value of 14000 was obtained.

DISCUSSION

The results show that the polysaccharide is not obviously polydisperse and the high limiting-viscosity number, and the marked dependence of the sedimentation constant on concentration suggest that the molecules are very asymmetrical. (Factors causing a dependence of sedimentation constant on concentration have been discussed by Kinell & Rånby (1950), and Ogston & Woods (1953).) The value of 90000 for the molecular weight calculated using the frictional ratio obtained from the viscosity measurements is regarded as being of the correct order of magnitude, whilst the figure of 14000 obtained assuming the molecule is spherical represents the minimum value. The effect of hydration could, however, reduce the higher figure. Few measurements have been made on similar polysaccharides from other bacteria, but Record & Stacey (1948) have reported molecular weights of 171×10^3, 540×10^3, and 141×10^3 for pneumococcus types I, II and III polysaccharides. Results obtained with other polysaccharides have been reviewed by Greenwood (1952).

SUMMARY

1. The capsular polysaccharide from an amylolytic rumen streptococcus has been found to be homogeneous in the ultracentrifuge.

2. The material possesses a high limiting-viscosity number and hence a very extended shape in solution (the frictional ratio $(f/f_0) = 3{\cdot}5$).

3. The molecular weight of 90000 calculated using the frictional ratio obtained from the viscosity measurements is regarded as being more correct than that calculated assuming a spherical molecule.

The author wishes to thank Prof. E. L. Hirst, F.R.S., for his interest in this work, and the Rockefeller Foundation for a gift of apparatus.

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Physicochemical Studies on Starches. Part I. The Characterization of the Starch present in the Seeds of the Rubber Tree, Hevea brasiliensis.

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The starch present in the endosperm of the seeds of the rubber tree, *Hevea brasiliensis*, is intimately associated with protein. Potentiometric iodine titrations on the purified material indicated that it contained 20% of amylose. Amylopectin (containing only 0·4—0·7% of amylose) and pure amylose fractions were obtained by fractionation of the starch with thymol followed by butan-1-ol. The average length of unit chain in the amylopectin was shown to be 23 ± 1 glucose residues by both methylation and periodate oxidation studies. The latter also indicated that the majority of interchain linkages were of the 1 : 6-type. Osmotic pressure measurements, on the acetylated derivative of the amylopectin in chloroform solution, indicated a molecular weight of about $1{\cdot}8 \times 10^6$ (*ca.* 6000 glucose residues). The acetylated amylose, prepared by a method involving the minimum of degradation, was shown similarly to possess a molecular weight of $4{\cdot}4 \times 10^5$ (*ca.* 1500 glucose residues). Measurements of the limiting viscosity number of both the free and the acetylated components were also carried out.

STARCH occurs in most seed materials, and many such sources have recently been examined. In this paper we report an investigation of the starch present in the endosperm of the seeds of the rubber tree, *Hevea brasiliensis*. It was isolated in granular form by a purely mechanical process avoiding reagents likely to cause degradation. It contained a large amount of protein (41%), which appeared to surround the granules. A modified Sevag technique (Sevag, *Biochem. Z.*, 1934, **273**, 419) reduced this in a portion of the starch to negligible amounts under the desired mild conditions. Portions of non-granular starch were obtained by further extraction of the starch–protein residue with chloral hydrate (Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 875) and then with hot water. Protein still contaminated the resultant products and had a marked effect on their interaction with iodine. [This is of general importance in the study of starch-like materials (Anderson and Greenwood, unpublished results).] The purified starch had a maximum binding power of 3·86% of iodine by weight, which under the conditions employed corresponded to an amylose-content of 20%.

A separation of the amylose and amylopectin fractions was carried out. The most satisfactory method for preparing purified amylopectin (0·4—0·7% of amylose) involved the use of thymol as a precipitant (Haworth, Peat, and Sagrott, *Nature*, 1946, **157**, 19), but yielded at the same time an impure amylose fraction (see Table 1). This fraction could then be purified *via* its butanol complex (Higginbotham and Morrison, *Shirley Inst. Mem.*, 1948, **22**, 148). To minimize inadvertent degradation of the linear component at elevated temperatures (Bottle, Gilbert, Greenwood, and Saad, *Chem. and Ind.*, 1953, 541), the fractionation was carried out as far as possible under nitrogen. Table 1 gives the results of potentiometric iodine titrations on, and measurements of the optical densities of the polysaccharide–iodine complexes (Hassid and McCready, *J. Amer. Chem. Soc.*, 1943, **65**, 1154) of, the fractionation products, in particular on material remaining in the supernatant liquors after removal of the various complexes. Amylose binding 19·2% of iodine by weight was obtained after five reprecipitations. Although it has been suggested that all amyloses possess the same maximum iodine-binding power (Higginbotham and Morrison, *loc. cit.*), recent work indicates that this may depend on the source of the amylose and the methods used for fractionation (see, *e.g.*, Schoch in Radley, " Starch and its Derivatives," Chapman & Hall, London, 1953, Vol. I, p. 123). The value will also depend on the

potentiometric titration conditions ([iodide ion], [iodine], pH, and temp.). For accurate results, it appears that the only satisfactory method for estimating the percentage of amylose in a given starch or its fractionation products involves an experimental determination of the maximum iodine-binding power of the *pure* amylose component of that starch and, if possible, the complexing reagent should be varied during this preparation. Percentages of amylose shown in Table 1 have been calculated on this basis. Fig. 1 shows the corresponding iodine titration curves. The weight of material in the supernatant liquor decreased regularly during a successful fractionation (if retrogradation occurred, the amount in the supernatant liquor increased and its properties changed). Amylopectin

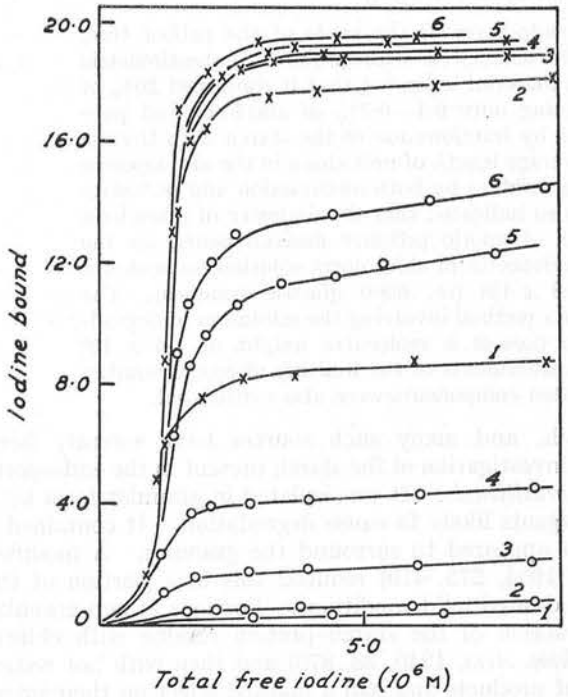

FIG. 1. *Iodine-titration curves of fractionation products.*
—— × —— *Curves for precipitates.*
—— ○ —— *Curves for material in corresponding supernatant liquor.*
1, *Thymol complex.*
2—6, *Butanol complexes* 1—5.

was obtained from the first butanol supernatant liquor, but the shape of the iodine-titration curves for subsequent supernatant liquors (Fig. 1) and the increase in the wave-length of maximum absorption of the polysaccharide–iodine complex (Table 1) suggested that after one or two re-fractionations actual molecular-weight fractionation of the amylose was occurring and short-chain amyloses were appearing in the supernatant liquors. The

TABLE 1. *Analyses of the fractionation products.*

Product		Wt. (mg.)	$\lambda_{max.}$ (Å)	Iodine affinity *	Amylose (%)
(a) *Precipitates.*					
Thymol complex		—	6000	8·3	43
Butanol complex :	1	—	—	17·4	91
	2	—	—	18·6	97
	3	—	—	18·8	98
	4	—	—	19·0	99
	5	227 †	6550	19·2 ‡	100
(b) *Material in supernatant liquors from* :					
Thymol complex		855	5500	0·14	0·7
Butanol complex :	1	230	5700	0·35	1·8
	2	30	6000	3·65	19
	3	19	—	1·70	9
	4	32	6250	10·6	55
	5	14	—	13·0	68

* Expressed as the maximum iodine binding power (%).
† A further 65 mg. were used in analyses of butanol complexes 1—4.
‡ Average of 6 determinations.

apparent molecular-weight distribution curve of the final amylose and the corresponding iodine affinity (cf. Kerr, Cleveland, and Katzbeck, *J. Amer. Chem. Soc.*, 1951, **73**, 3916) may thus depend on the method of fractionation. In this instance, 7% of the total weight of starch was present in the supernatant liquors. (This is increased to 20% if that from the first butanol complex is included.) Fractionation involving the use of thymol followed by butanol as complexing agents appears to be a general method for preparation of both pure amylose and amylopectin (Anderson and Greenwood, unpublished observations).

Methylation of the amylopectin fraction was carried out by suspending the polysaccharide in liquid ammonia and treating the suspension with sodium and methyl iodide under the conditions described by Hodge, Karjala, and Hilbert (*ibid.*, p. 3312). This method was claimed to give completely methylated starch products without severe degradation. The trimethyl derivative was treated with methanolic hydrogen chloride, and the methylated methyl glucosides obtained were hydrolysed with aqueous hydrochloric acid. Examination of the mixture of reducing sugars both on paper chromatograms and on a column of powdered cellulose showed the presence of 2 : 3 : 4 : 6-tetra-*O*-methyl-, 2 : 3 : 6-tri-*O*-methyl-, and 2 : 3-di-*O*-methyl-glucose, and a mixture of 2 : 6- and 3 : 6-di-*O*-methylglucose, which probably arose from incomplete methylation or some demethylation during hydrolysis. Only traces of mono-*O*-methylglucoses and glucose were detected.

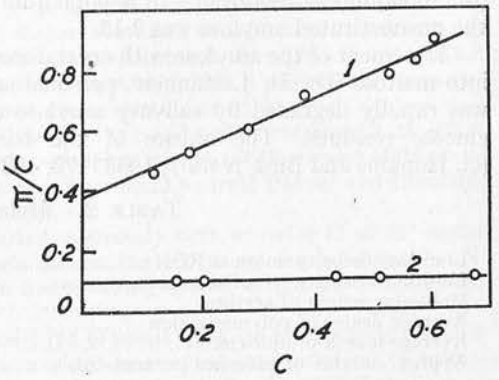

FIG. 2. *Graph of* π/c versus c *for the acetylated starch components in chloroform solution.*

1, *Amylose acetate.*
2, *Amylopectin acetate.*

The yield of tetra-*O*-methylglucose corresponded to the presence of one non-reducing terminal group for every twenty-three glucose residues in the molecule. The value was confirmed by periodate oxidation of both the amylopectin fraction and the whole starch. It follows that the average length of unit chain in the amylopectin is 23 glucose residues, a value similar to that found in other starches (cf. Brown, Halsall, Hirst, and Jones, *J.*, 1948, 27). Further, the isolation of only 0·75% of glucose from the hydrolysis products of the periodate-oxidized amylopectin showed that the majority (80%) of the interchain linkages were of the 1 : 6-type.

As a preliminary to determining the size of the amylopectin, the tri-*O*-acetyl derivative was prepared. Difficulty was found in carrying out this esterification with a pyridine–acetic anhydride mixture even on freeze-dried material, although various authors have claimed this to be successful (Higginbotham and Morrison, *loc. cit.*; Meyer, Bernfeld, and Hohenemser, *Helv. Chim. Acta*, 1940, **23**, 885). Potter and Hassid's method (*J. Amer. Chem. Soc.*, 1948, **70**, 3774) involving a prior dispersion in formamide was the most satisfactory. The limiting viscosity number $[\eta]$ (I.U.P.A.C. nomenclature, *J. Polymer Sci.*, 1952, **8**, 257) of the triacetate in chloroform solution was 2·33. Measurements of the osmotic pressure of the acetate in chloroform solution indicated that the number-average molecular weight ($\bar{M}_n$) was approximately 1,800,000. The π/c *versus* c curve (Fig. 2) appeared to be linear for the range of concentrations investigated (cf. Kerr, Cleveland, and Katzbeck, *J. Amer. Chem. Soc.*, 1951, **73**, 111). The molecular-weight data, in conjunction with the average length of unit chain of 23 glucose residues, showed that the amylopectin had a highly branched structure (*ca.* 260 branches per molecule). The amylopectin was also

characterized by measuring the limiting viscosity number of the unsubstituted component in M-potassium hydroxide (Lansky, Kooi, and Schoch, *ibid.*, 1949, **71**, 4066). For comparison, the limiting viscosity number of a sample of rabbit-liver glycogen (kindly provided by Dr. D. J. Manners) was measured in this solvent.

Methylation of the amylose was not attempted. Although other workers have isolated a small quantity of tetra-*O*-methylglucose from the hydrolysed tri-*O*-methyl derivative (see, *e.g.*, Mayer, Wertheim, and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 865; Bourne, Fantes, and Peat, *J.*, 1949, 1109; MacWilliam and Percival, *J.*, 1951, 2259), the accuracy of such an estimation is not high with limited quantities of material. In view of this, and the labile nature of the linkages in the linear molecule (Bottle, Gilbert, Greenwood, and Saad, *loc. cit.*), it appeared most satisfactory to estimate the chain length of the amylose from the molecular size of the tri-*O*-acetyl derivative, although this procedure cannot indicate whether the molecule is entirely linear (cf. Kerr and Cleveland, *J. Amer. Chem. Soc.*, 1952, **74**, 4036; Potter and Hassid, *loc. cit.*). An estimate of the degradation accompanying acetylation of amylose was obtained by measuring the limiting viscosity numbers of products isolated under different reaction conditions (see Table 3), and the method involving minimum degradation was then used for esterification. The limiting viscosity number of the product was 3·35, whilst the number-average molecular weight obtained from osmotic-pressure measurements in chloroform solution (Fig. 2) was 440,000 (*ca.* 1500 glucose residues). In M-potassium hydroxide, the limiting viscosity number for the unsubstituted amylose was 2·15.

Treatment of the amylose with crystalline sweet potato β-amylase gave 79% conversion into maltose (Dr. D. J. Manners, personal communication). The residual polysaccharide was rapidly degraded by salivary amylase and was therefore composed of α-1 : 4-linked glucose residues. The nature of the barrier to β-amylolysis is being investigated (cf. Hopkins and Bird, *Nature*, 1953, **172**, 492; Peat and Whelan, *ibid.*, p. 494).

TABLE 2. *Molecular-weight data.*

	Amylose	Amylopectin	Glycogen
Limiting viscosity no. in M-KOH	2·15	1·02	0·10
Limiting viscosity no. of acetate	3·35	2·33	—
Molecular weight of acetate	$4{\cdot}4 \times 10^5$	$1{\cdot}8 \times 10^6$	$4{\cdot}8 \times 10^6$ *
Average degree of polymerization	1,500	6,000	30,000
Average length of unit-chain	—	23	12
Approx. number of branches per molecule	—	260	2,500

* From sedimentation measurements on unsubstituted glycogen (Greenwood and Manners, unpublished results).

The molecular-weight data are summarized in Table 2. The values are of the same order as those reported for the components from other starches (see, *e.g.*, Potter and Hassid, *loc. cit.*; Potter, Hassid, and Joslyn, *J. Amer. Chem. Soc.*, 1949, **71**, 4075; Higginbotham, *Shirley Inst. Mem.*, 1950, **24**, 221).

Although kinetic-energy corrections have been considered in obtaining the limiting viscosity numbers quoted, no correction was applied for shear effects (cf. Krigbaum and Flory, *J. Polymer Sci.*, 1953, **11**, 37). These may be considerably more important in the case of the elongated amylose component than for the branched products. Experiments are now in progress to investigate this. The large difference between the limiting viscosity numbers of the amylopectin and the glycogen samples is significant, and must be related to a fundamental difference in the molecular structure of these branched α-1 : 4-glucosans (cf. Hirst and Young, *J.*, 1939, 1471), as a result of which the amylopectin molecule must possess a more extended shape in solution than glycogen. It has been suggested recently by Hirst and Manners (*Chem. and Ind.*, 1954, 224) that this is due to the two polysaccharides' having different degrees of multiple branching.

EXPERIMENTAL

Preliminary Extraction of Oil from the Rubber Seed Endosperm.—Crushed endosperm was exhaustively extracted with benzene–methanol (1 : 1, v/v). The product was filtered, dried *in vacuo* over calcium chloride, and then ground in a hammer-mill to yield a fine buff-coloured powder (47% of the original weight of endosperm).

Isolation of Starch.—Defatted endosperm (600 g.) was extracted several times (*ca.* 24 hr. periods) by shaking it with cold water under toluene (6 l.), and the crude starch was isolated by filtering the aqueous suspension through muslin. The filtrate yielded a light brown sediment, which was washed by decantation with water and then refluxed three times with 80% aqueous methanol (30 ml./1 g.; 3 hr. periods) to ensure complete removal of fats. The yield was *ca.* 60 g. [Found: N (semi-micro Kjeldahl), 6·5%; *i.e.*, protein, 40·6%].

Removal of Protein from the Starch.—Microscopic examination indicated that contaminating protein probably formed a surface layer, as little extraneous material was visible and the granules were noticeably larger than in the purified starch (apart from the difference in size, little change in outward appearance was evident). Treatment with water at 50° did not coagulate the protein, but leached material giving a blue stain with iodine from the granule.

(a) *Treatment with toluene and butanol.* A suspension of starch in M-sodium chloride (2 g./200 ml.) was shaken with an equal volume of toluene for 6 hr. to denature the protein, and then set aside for 3 hr. to allow separation of the starch granules from the toluene–protein layer. (This procedure gave more effective separation than centrifugation at low speeds.) The brown precipitate obtained at the toluene–water interface was removed. The sedimented product consisted of two layers, the lower being mainly starch and the brown upper one mainly protein. These two layers were then each resuspended in M-sodium chloride and re-extracted with toluene as above. Combining the lower white layers, and also the upper layers, and continually re-extracting them with toluene, gave three products: (i) white starch which formed no interfacial precipitate, (ii) pale brown impure starch, and (iii) coagulated protein. Each was then suspended in M-sodium chloride (1 vol.) and shaken overnight with butanol (2·5 vols.), and the above procedure repeated. The products, after removal of salt by repeated decantation with water, consisted of starch A (*ca.* 15 g.) (Found: N, 0·05%) and a protein-contaminated residue. Starch A was never dried and was stored under ethanol at 0°.

(b) *Extraction with chloral hydrate.* Starch in the residue could not be extracted with either (i) 1% sodium sulphite solution or (ii) 0·3% aqueous sulphur dioxide (Hilbert and McMasters, *J. Biol. Chem.*, 1946, **162**, 229) and so was extracted with chloral hydrate (Mayer and Bernfeld, *loc. cit.*), to yield starch B (5·2 g.) (Found: N, 0·41%).

(c) *Hot-water extraction.* The residue was stirred vigorously with water (2 l.) at 95° under nitrogen for 1 hr. The protein residue was removed on the centrifuge (18 g.) (Found: N, 14·4%), and the supernatant liquor yielded on freeze-drying starch C (20 g.) (Found: N, 4·9%).

Properties of Starch A.—This white powder consisted of small spherical birefringent granules (diam. 4·4 μ). In hot water it formed a clear paste which gave a deep blue colour with iodine. On hydrolysis with 2% sulphuric acid, the starch yielded 98% of the theoretical amount of glucose (quantitative chromatographic determination), and no other sugar could be detected on the paper chromatogram. This material had $[\alpha]_D^{16}$ +161° (*c*, 0·763% in N-NaOH), $[\alpha]_D^{16}$ +156° (*c*, 1·0% in 30% $HClO_4$) (Found: ash, 0·05; N, 0·05%). The optical density of the colour developed when starch (1 mg.) was stained with iodine (2 mg.) and potassium iodide (20 mg.) in distilled water (100 ml.) was measured at various wave-lengths between 4000 and 7000 Å in cells of 1 cm. length against the same iodine solution (Hassid and McCready, *loc. cit.*) by means of a Unicam spectrophotometer. For comparison with "blue values" (Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924), optical densities in a 4-cm. cell at 6800 Å were calculated by assuming a linear relation between optical density and (*a*) length of cell and (*b*) concentration at this wave-length. For starch A, this density was 0·31 (the corresponding value for starch B was 0·27).

Potentiometric Iodine Titration.—The differential potentiometric titration technique of Gilbert and Marriott (*Trans. Faraday Soc.*, 1948, **44**, 84) was employed, with the electrometer described by Anderson and Greenwood (*Chem. and Ind.*, 1953, 476). The sensitivity of this electrometer enabled potentials of 0·01 mv to be measured. Titration conditions were: [iodide], 0·01M; [starch], *ca.* 1 mg./50 ml.; [amylose], *ca.* 1 mg./200 ml.; [amylopectin], *ca.* 1 mg./30 ml.; pH, 5·85; temp., 20°. The two half-cells were stirred automatically throughout the experiment. With careful mixing of solutions, a negligible potential difference was observed at the start of the experiment. Points on the titration curve were obtained by adding small increments of iodine to the solution cell, and then adding iodine to the control until the concentration of free iodine in each cell was equal after 2—3 min. had been allowed for equilibration. The total free iodine in the starch solution was plotted against mg. of iodine bound per 100 mg. of polysaccharide. Starch A showed a maximum binding power of 3·86%, which under the conditions used corresponded to an amylose content of 20% (see below).

Starch B had a binding power of 3·02% (15·7% of amylose), which suggested that protein present interfered considerably with the iodine uptake of the starch.

Starch A was used in all further investigations.

Periodate Oxidation of the Starch.—A sample (0·469 g.) in water (80 ml.) was treated with 0·25M-sodium metaperiodate (40 ml.) and potassium chloride (5 g.) as described by Brown, Halsall, Hirst, and Jones (*J.*, 1948, 27). The yield of formic acid corresponded to 0·034 mole from 162 g. of the starch after 220 hr. (constant value), *i.e.*, 1 mole per 24 glucose residues in the amylopectin fraction. A duplicate experiment yielded 1 mole per 23 glucose residues.

Determination of Glucose Residues in the Starch linked through $C_{(1)}$, $C_{(4)}$, *and* $C_{(6)}$.—After completion of the above oxidation the solutions were freed from periodate and hydrolysed, and the percentage of glucose remaining was quantitatively estimated by MacWilliam and Percival's method (*J.*, 1951, 2259). The amount (1·2%) of glucose found indicated that 98·8% of the residues in the starch were linked through $C_{(1)}$–$C_{(4)}$ or $C_{(1)}$–$C_{(6)}$, and that, as the chain length was 24 glucose units, about 70% of the interchain linkages were of the 1 : 6-type.

Fractionation of the Starch.—The starch was defatted by refluxing it several times with methanol, and then portions were fractionated with thymol as a complexing agent (Haworth, Peat, and Sagrott, *loc. cit.*), followed by butanol (Higginbotham and Morrison, *loc. cit.*). Fractionation was carried out under nitrogen to minimise degradation of amylose (Bottle, Gilbert, Greenwood, and Saad, *loc. cit.*). The course of the fractionation was followed by potentiometric and optical measurements on the polysaccharide–iodine complexes of the precipitated material, and in addition on material in the supernatant liquors.

(a) *Fractionation I.* This will not be reported in detail. Starch (8 g.) yielded amylose I [*ca.* 1·70 g.; $O.D._{4\ cm.}^{6800}$ (*i.e.* optical density as above) 1·00; $\lambda_{max.}$ 6250 Å; max. iodine-binding power, 15·4%], amylopectin I (4·3 g.; $O.D._{4\ cm.}^{6800}$ 0·08; $\lambda_{max.}$ 5250 Å; max. iodine-binding power, 0·07%; amylose, 0·4%), and supernatant material (1·60 g.). Improvements in methods were incorporated in fractionation II.

(b) *Fractionation II.* A suspension of starch (*ca.* 2 g.) in water (40 ml.) was added to vigorously stirred boiling water (260 ml.) under nitrogen, and boiling continued for 20 min. The temperature was then allowed to fall to 60°, powdered thymol (0·5 g.) was added, and the mixture stirred at 60° for 30 min., then kept at room temperature (15—17°) for 3 days to allow the thymol complex to be precipitated. The suspension was then passed three times through a Sharples supercentrifuge to remove the amylose complex as completely as possible. The clear supernatant liquor was freeze-dried, refluxed with methanol to remove thymol, redissolved, and freeze-dried again, to yield amylopectin II (1·38 g.; $O.D._{4\ cm.}^{6800}$ 0·08; max. iodine-binding power, 0·13%; amylose, 0·7%). The thymol–amylose complex was directly dispersed in boiling water (200 ml.; under nitrogen), and the amylose reprecipitated by saturating the solution with butanol of analytical quality (20 ml.; redistilled over sodium hydroxide), stirring at 95° for ½ hr., and then allowing the solution to cool slowly to room temperature (in a Dewar flask, 24 hr.). The resultant butanol complex was removed on the supercentrifuge, and then redispersed and reprecipitated with butanol. After four butanol re-fractionations the amylose obtained (II*a*) was stored as its butanol complex (*ca.* 240 mg.; $O.D._{4\ cm.}^{6800}$ 1·21; max. iodine-binding power, 18·7%). 250 mg. of material were obtained when the clear supernatant liquors from each reprecipitation were freeze-dried. Total recovery was approximately 90%, losses being purely mechanical.

To check the value of the limiting iodine-binding power of the amylose, the whole of the above fractionation procedure was repeated with 800 ml. of water to disperse the starch (*ca.* 1·5 g.), and re-fractionation from 400 ml. of water saturated with butanol. Analyses of the fractionation products are given in Table 1. Amylose II*b* was isolated by stirring the butanol complex several times with butanol and then drying it *in vacuo* at 75° (Schoch, *loc. cit.*).

The amylopectin fractions were used without further purification.

Examination of the amylopectin.

Determination of Non-reducing End-group by Periodate Oxidation.—The method described above was used. 0·048 mole of formic acid liberated from 162 g. of amylopectin corresponded to one non-reducing terminal end-group per 23 glucose residues.

Determination of Glucose Residues linked through $C_{(1)}$, $C_{(4)}$, *and* $C_{(6)}$.—0·75% of glucose isolated as above from the periodate-oxidized amylopectin indicated that 82% of the interchain linkages were of the 1 : 6-type.

Methylation of the Amylopectin.—The modification by Hodge, Karjala, and Hilbert (*loc. cit.*) of the liquid-ammonia method of Freudenberg, Boppel, and Meyer-Delius (*Naturwiss.*, 1938,

26, 123) was used. Dry liquid ammonia, and a reaction temperature of −30°, were essential. After every four additions of reagents the product was isolated, dialysed, and freeze-dried before continuing the treatment. The sample (3 g.) was treated with seventeen additions of reagents, after which the methylated product (2·7 g.) was isolated (Found: OMe, 43·5%), having $[\alpha]_D^{16}$ +204° (*c*, 0·5 in $CHCl_3$). Further methylation was carried out by Purdie and Irvine's method (*J.*, 1903, 1021). The product (2·1 g.) was purified by precipitation from chloroform solution with light petroleum (b. p. 40—60°) (Found: OMe, 43·8. Calc. for tri-*O*-methylamylopectin: OMe, 45·6%).

Hydrolysis of Methylated Amylopectin.—(*a*) The sample (50 mg.) was hydrolysed in a sealed tube by the method of Hough, Hirst, and Jones (*J.*, 1949, 928). The hydrolysate was examined by paper-strip chromatography with butanol–ethanol–water (4 : 1 : 5, v/v) as the mobile phase, and revealed 2 : 3 : 4 : 6-tetra-*O*-methyl- (R_G 1·0 (4·8%), 2 : 3 : 6-tri-*O*-methyl- (R_G, 0·83) (85·0%), di-*O*-methyl- (R_G, 0·59, 0·51) (9·5%), and a trace of mono-*O*-methyl-glucose and glucose. This result indicated the presence of one non-reducing terminal group per 22 ± 1 glucose residues.

(*b*) The material (1·8 g.) was heated with methanolic hydrogen chloride (110 ml.; 1%) for 5 hr. at 100°, then neutralized with silver carbonate, washed, freed from excess of silver with hydrogen sulphide, and concentrated. The syrupy glycosides obtained were boiled with 2% hydrochloric acid (88 ml.) for 8 hr. The solution was then neutralized with silver carbonate as above, deionized by shaking it with resins, and concentrated to give a clear syrup (1·75 g., 95%). This mixture of methylated glucoses was separated on a column (64 × 3 cm.) of cellulose (Hough, Jones, and Wadman, *J.*, 1949, 2511; Chanda, Hirst, Jones, and Percival, *J.*, 1950, 1289). Elution with butanol–light petroleum (b. p. 100—120°) (3 : 7, v/v) saturated with water gave fractions (1) 0·242 g., (2) 1·172 g., and (3) 0·139 g. By elution with butanol a further fraction (4) 0·058 g. was obtained, whilst elution with water gave a trace of a fraction, which from examination on a paper chromatogram, was mono-*O*-methylglucose and glucose. The last fraction was not examined further.

Examination of the fractions. Fraction (1). Examination by paper-strip chromatography indicated the presence of a single sugar (R_G, 1·0) corresponding to 2 : 3 : 4 : 6-tetra-*O*-methyl-glucose. Hydrolysis of a small portion (10 mg.) with 2% sulphuric acid, and re-examination revealed an additional substance (R_G, 0·84). Hypoiodite oxidation indicated that fraction (1) contained only 30% of tetra-*O*-methylglucose. Fraction (1) (210 mg.) was then rehydrolysed at 100° with 1% hydrochloric acid (20 ml.) for 5 hr., and the methylated glucoses were separated on Whatman 3MM papers with benzene–ethanol–water (149 : 45 : 15, v/v) as the mobile phase for 4 hr. Elution gave fractions (1*a*) (59 mg.) and (1*b*) (197 mg.) (94% recovery). Hypoiodite oxidation indicated that fraction (1*a*) contained 99% of tetra-*O*-methylglucose. After being recrystallized twice from light petroleum (b. p. 40—60°), the material (50 mg.) had m. p. 85—87°, which was not depressed on admixture with an authentic specimen of tetra-*O*-methyl-D-glucopyranose; $[\alpha]_D^{16}$ +85° (final) (*c*, 0·4 in H_2O) (Found: OMe, 52·0. Calc. for $C_{10}H_{20}O_6$: OMe, 52·5%). The anilide had m. p. 136—138°, alone or mixed with authentic tetra-*O*-methyl-D-glucopyranosylaniline (Found: N, 4·3; OMe, 39·5. Calc. for $C_{16}H_{25}O_5N$: N, 4·5; OMe, 39·9%). From the above results the amount of 2 : 3 : 4 : 6-tetra-*O*-methylglucose was calculated to be 72·3 mg., corresponding to one non-reducing terminal group per 23 glucose residues.

Fraction (1*b*) was shown to consist of a single substance (R_G 0·84) corresponding to 2 : 3 : 6-tri-*O*-methylglucose. It was crystallized from ether and had m. p. 115—117° (alone or mixed with an authentic specimen of 2 : 3 : 6-tri-*O*-methylglucose), $[\alpha]_D^{17}$ +70° (*c*, 0·4 in H_2O), $[\alpha]_D^{17}$ +65° (initial), −35° (final) [*c*, 0·4 in cold methanolic hydrogen chloride (2% for 10 hr.)] (Found: OMe, 41·3. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction (2). Paper chromatography indicated that this fraction consisted of one component (R_G 0·84), corresponding to 2 : 3 : 6-tri-*O*-methylglucose. It had m. p. 115—117° (alone or mixed with authentic 2 : 3 : 6-tri-*O*-methyl-D-glucose), $[\alpha]_D^{17}$ +68° (*c*, 1·0 in H_2O), $[\alpha]_D^{17}$ +67° (initial), −36° (final) [*c*, 1·0 in cold methanolic hydrogen chloride (2%, for 10 hr.)] (Found: OMe, 41·5. Calc. for $C_9H_{18}O_6$: OMe, 41·9%).

Fraction (3). Chromatographic examination of this fraction revealed the presence of two substances; one (R_G 0·84) corresponding to 2 : 3 : 6-tri-*O*-methylglucose, the other (R_G 0·59) to 2 : 3-di-*O*-methylglucose. When separated on Whatman 3MM papers and extracted with acetone, a portion gave fractions (3*a*) (18·8 mg.) and (3*b*) (75 mg.) (94% recovery). Fraction (3*a*) was shown to be 2 : 3 : 6-tri-*O*-methylglucose as above. Fraction (3*b*) was chromatographically pure and was a pale yellow syrup, which did not crystallize after two

weeks in the cold. It had $[\alpha]_D^{17}$ +106° (initial), +68° (final) (*c*, 1·0 in H_2O) (Found: OMe, 29·3. Calc. for $C_8H_{16}O_6$: OMe, 29·7%). The di-*O*-methylglucose was converted into the gluconolactone and then into 2 : 3-di-*O*-methylgluconophenylhydrazide (Evans, Levi, Hawkins, and Hibbert, *Canad. J. Res.*, 1942, **20**, *B*, 175). The product had m. p. 160—162° (Evans *et al.* give 166·5—167°) (Found: OMe, 19·4; N, 9·0. Calc. for $C_{14}H_{22}O_6N_2$: OMe, 19·7; N, 8·9%).

Fraction (4) was shown chromatographically to consist of 2 : 6- or 3 : 6-di-*O*-methylglucose, or a mixture of the two (R_G 0·51). When it was rehydrolysed and re-examined no other sugar was detected. The fraction was a yellow syrup, which failed to crystallize during two weeks in the cold and had $[\alpha]_D^{17}$ +78° (initial), +60° (final) (*c*, 0·5 in H_2O), $[\alpha]_D^{17}$ +60° (initial), −10° (final) [*c*, 0·5 in methanolic hydrogen chloride (2%; 10 hr. const.)] (Found: OMe, 29·2. Calc. for $C_8H_{16}O_6$: OMe, 29·7%). The uptake of periodate (Bell's method, *J.*, 1948, 992) indicated that 64% of the fraction was 2 : 6-di-*O*-methyl-D-glucose. When excess of periodate was destroyed, the solution evaporated to dryness, and the chloroform-soluble material examined chromatographically, 34% of 3 : 6-di-*O*-methylglucose was found.

Acetylation of the Amylopectin.—Amylopectin (245 mg.) was acetylated with formamide as a dispersive agent (Potter and Hassid, *loc. cit.*). Purification by precipitation from chloroform with light petroleum (b. p. 80—100°) gave a fibrous product (Ap 1) (370 mg., 92%) (Found: Ac, 44·2. Calc. for amylopectin tri-*O*-acetate: Ac, 44·8%), $[\eta]$ 2·33 (in $CHCl_3$).

Examination of the amylose.

Degradative Effect of Acetylation.—Portions of amylose 1 were acetylated by Higginbotham and Morrison's method (*loc. cit.*) at (*a*) room temperature (16°) for 74 and 186 hr. to yield acetates (A 1) and (A 2) and (*b*) 100° for 4 and 8 hr. to yield acetates (A 3) and (A 4) respectively. The products were purified as above. Acetate A 1 (54 mg.) (Found: Ac, 44·3. Calc. for amylose tri-*O*-acetate: Ac, 44·8%) had $[\eta]$ 2·63 in $CHCl_3$. Acetate A 2 (52 mg.) (Found: Ac, 43·9%) had $[\eta]$ 2·45. Acetate A 3 (47 mg.) (Found: Ac, 44·1%) had $[\eta]$ 2·55. Acetate A 4 (42 mg.) (Found: Ac, 44·0%) had $[\eta]$ 2·38. When the limiting viscosity numbers $[\eta]$ were plotted against the time of reaction (*t*), the rate of breakdown was so small that it was assumed legitimate to extrapolate the $[\eta]$–*t* curve to zero time to obtain the limiting viscosity number of the undegraded amylose. This enabled the rate of degradation to be calculated and showed that minimum degradation occurred at room temperature in the time required to give complete acetylation.

Molecular weights (*M*) and corresponding degrees of polymerization (D.P.) were later calculated from the equation $M = 1{\cdot}3 \times 10^5 \times [\eta]$ (where $[\eta]$ is the limiting viscosity number) obtained from the osmotic pressure measurements, as shown in Table 3.

TABLE 3. *Results of viscosity measurements.*

Product	Acetylation time (hr.)	$[\eta]$	$10^{-5}M$	Approx. D.P.	Rate of degradation *
A 1	74	2·63	3·42	1190	0·001
A 2	146	2·45	3·19	1110	0·001
A 3	4	2·55	3·32	1150	0·025
A 4	8	2·38	3·10	1080	0·023
—	0	2·80 †	3·64 †	1260 †	—

* Expressed as the number of bonds broken per initial amylose molecule per hr.
† Extrapolated values.

Acetylation of Amylose 2a.—Amylose–butanol complex (containing *ca.* 240 mg. of amylose) was acetylated as above for three days at room temperature, to yield amylose acetate (A 5) (400 mg.) (Found: OAc, 44·3%), $[\eta]$ 3·35.

Physical measurements.

Measurements of Limiting Viscosity Number.—The limiting viscosity number $[\eta]$ for the acetylated products was determined from the relation $[\eta] = \lim_{c \to 0} (\eta_{sp.}/c)$. Specific viscosities ($\eta_{sp.}$) in chloroform solution were determined with a modified Ubbelohde viscometer (Davis and Elliott, *J. Coll. Sci.*, 1949, **4**, 313) at 22·5° ± 0·001°. Solvent flow time was 192·0 sec. By measuring the viscosity of a series of liquids, the kinetic-energy correction factor *B* in the equation $\eta = Adt + Bd/t$ (d = density of liquid) for a capillary viscometer was shown to be zero. The viscosity of the most concentrated solution was measured first, and the dilutions were made *in situ* in the viscometer. Final concentrations (*c*) were determined in duplicate by

the method described by Gilbert, Graff-Baker, and Greenwood (*J. Polymer Sci.*, 1951, **6**, 585) and were expressed as g. per 100 ml. of solution. Results were as tabulated.

Product	c	$\eta_{sp.}$	$\eta_{sp.}/c$	Product	c	$\eta_{sp.}$	$\eta_{sp.}/c$
A 1	0·201	0·705	3·51	A 2	0·210	0·626	2·98
	0·134	0·436	3·25		0·140	0·399	2·85
	0·100	0·307	3·06		0·105	0·286	2·73
	0	—	2·63 *		0	—	2·45 *
A 3	0·186	0·568	3·05	A 4	0·200	0·536	2·85
	0·124	0·375	3·02		0·133	0·336	2·68
	0·093	0·274	2·95		0·100	0·258	2·58
	0	—	2·55 *		0	—	2·38 *
A 5	0·575	2·376	4·13	Ap 1	0·540	1·568	2·90
	0·384	1·533	3·99		0·360	0·956	2·68
	0·287	1·102	3·84		0·270	0·675	2·50
	0·230	0·839	3·65		0·216	0·547	2·53
	0·192	0·687	3·58		0·180	0·443	2·46
	0·143	0·522	3·65		0·135	0·340	2·52
	0·115	0·463	3·53		0·108	0·263	2·40
	0	—	3·35 *		0	—	2·33 *

* Extrapolated values.

Measurements were also carried out on the unsubstituted components dissolved in M-potassium hydroxide. The viscometer used had a solvent flow time of 227 sec., and no kinetic-energy correction was necessary. Solutions were prepared by shaking polysaccharide (50—100 mg.) in M-potassium hydroxide (15 ml.) vigorously at room temperature for 30 min. Before being placed in the viscometer, each solution was filtered under gravity through sintered glass (G3, then G4). The results, which were reproducible in the short time required for dissolution and measurement, are tabulated. Measurements on a sample of rabbit-liver glycogen in the same solvent are also included.

Product	c	$\eta_{sp.}$	$\eta_{sp.}/c$	Product	c	$\eta_{sp.}$	$\eta_{sp.}/c$
Amylose	0·333	0·960	2·88	Amylopectin ...	0·474	0·766	1·62
	0·222	0·585	2·64		0·316	0·443	1·41
	0·167	0·417	2·50		0·237	0·313	1·32
	0·111	0·265	2·39		0·158	0·193	1·22
	0	—	2·15 *		0	—	1·02 *
Glycogen	0·666	0·073	0·110				
	0·333	0·035	0·105				
	0·267	0·029	0·108				
	0·167	0·017	0·102				
	0	—	0·100 *				

* Extrapolated values.

Measurement of Osmotic Pressure (with W. N. BROATCH).—Osmotic pressures (π) of amylose acetate (A5) in chloroform were determined at 22·5°. The osmometer used was a modified Fuoss–Mead instrument (*J. Phys. Chem.*, 1943, **47**, 59) designed for complete immersion in a thermostat (cf. Masson and Melville, *J. Polymer Sci.*, 1949, **4**, 323). The membrane was of gel-cellophane (No. 600) conditioned to solvent after dehydration with acetone–water mixtures. It was held taut in the osmometer by rings (cf. Gilbert, Graff-Baker, and Greenwood, *loc. cit.*). Observed osmotic pressures (measured to 0·001 cm.) were corrected for the solution density (*idem, loc. cit.*). Density determinations showed that this correction was $-0{\cdot}002\ h_s c$ (h_s = height from the mid-point of the vertical membrane to the solution meniscus; c = concentration), and the results shown in Fig. 2 include this. Concentrations were estimated in duplicate as above.

Measurements on amylopectin acetate (Ap 1) were made in the osmometer previously described (*idem, loc. cit.*), in which for greater ease in manipulation, the solvent capillary diameter was altered to 0·04 cm. The membrane was as above. The density correction was $+0{\cdot}003\ h_s c$, and is included in the results shown in Fig. 2.

In each experiment, the value of the intercept $(\pi/c)_0$ was obtained from the graph of π/c versus c and was found to be 0·380 and 0·097 for amylose acetate and amylopectin acetate, respectively. Substitution of these values in van't Hoff's equation gave the number-average molecular weights ($\bar{M}_n$), since $\bar{M}_n = \boldsymbol{R}T(c/\pi)_0/100d_0\boldsymbol{g}$ ($\boldsymbol{R} = 8{\cdot}315 \times 10^7$ ergs/degree/mole; $\boldsymbol{g}$ = gravitational constant; and d_0 = density of chloroform at 22·5°). The value of $\bar{M}_n$ was $4{\cdot}4 \times 10^5$ for A 5 and $1{\cdot}8 \times 10^6$ for Ap 1.

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CONTENTS

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Observations on Some Possible Errors in Osmometry

Number-average molecular weights ($\bar{M}_n$) obtained from osmotic pressure measurements for a given sample often show variation.[1] In a recent communication, Cleverdon and co-workers[2] have discussed some of the possible causes for the discrepancy between the value of $\bar{M}_n$ found by them and by Hookway and Townsend[3] for a broad fraction of commercial polystyrene. Through the courtesy of Dr. D. Cleverdon we have also been able to examine this sample, and wish to report briefly our observations.

Osmotic pressures of solutions of the polystyrene in chloroform were measured using an instrument previously described[4] (for the loan of which we are indebted to Dr. G. A. Gilbert), in which the diameter of the solvent capillary had been increased to 0.04 cm. The cell constant was then independent of the meniscus level and was reproducible within the setting of the cathetometer (±0.001 cm.). In addition, one measurement was made in a Fuoss-Mead instrument, modified for complete immersion[5] and fitted with 0.1 cm. diameter capillaries, in which the cell constant was negligible (±0.001 cm.). The membranes used were of No. 600 gel-cellophane, which had been dehydrated by acetone and then conditioned to solvent. Pressures were measured statically after an initial setting to within −0.1 cm. of the expected value. Although gel-cellophane membranes have been described as truly semipermeable down to $\bar{M}_n \approx 10{,}000$,[6] solute permeation was found to occur in both osmometers, which suggested that the sample was indeed a very broad fraction. The initial rate of decrease in the static pressure head was *ca.* 1.0%/hr. in the Fuoss-Mead instrument, and *ca.* 0.3%/hr. in the other osmometer, and when correcting for this it was assumed legitimate to extrapolate pressures linearly to zero time (0.04% solute permeated into the solvent chamber when a solution containing 1.257 g./100 ml. was left for 72 hr. in the Fuoss-Mead instru-

TABLE I

OSMOTIC PRESSURE MEASUREMENTS FOR POLYSTYRENE SAMPLE IN CHLOROFORM SOLUTION AT 22.5°C.

Concentration (C) in g./100 ml. solution	Osmotic pressure (π) in cm. solvent	π/C
1.257	2.596	2.07[a]
0.956	1.682	1.76
0.766	1.238	1.62
0.487	0.641	1.32
0.443	0.569	1.28
0.358	0.413	1.15
0.270	0.280	1.04
0.165	0.169	1.02
0.079	0.075	0.95
0	—	(0.86)[b]

[a] Measurement in the Fuoss-Mead osmometer.
[b] By method of least squares.

ment). The results, shown in Table I, include corrections for the density of solution.[5] The π/C *vs.* C curve appeared linear, and application of the method of least squares showed that the data were best represented by the equation:

$$\pi/C = 0.861 + 0.952\ C$$

(The standard deviation in π/C was 0.023.)

These figures give a value of 195,000 ± 5,000 for $\bar{M}_n$, which may be compared with 222,500 and 210,000 obtained using denitrated collodion membranes in Fuoss-Mead instruments[2] and 156,500 using polyvinyl alcohol membranes in a Zimm-Myerson instrument.[3] Although the latter values were from measurements in benzene solution, a value of 210,000 has been obtained in chloroform solution by Bawn.[7] Possible causes of discrepancy are (*1*) solute permeation, (*2*) solute adsorption on the membrane, (*3*) membrane "dissymmetry" effects, and (*4*) neglect of an appropriate correction for the density of the solution.

In our measurements, although solute permeation was apparent, a correction has been applied for it. The consistent results from the two osmometers suggested furthermore that this correction was adequate. The effect of solute permeation is far more noticeable in either of the instruments used here than it would be in a Zimm-Myerson osmometer, owing to the different solvent volumes used. There was no evidence of membrane dissymmetry. The effect of adsorption of solute may well vary with the type of membrane used. In our experiments, the effect on the observed osmotic pressures of any random adsorption of solute (*i.e.*, "nonselective" adsorption) on the membrane was eliminated, as all concentrations were measured (in duplicate) *after* each determination using the method previously described.[5] Proof of the absence of any preferential adsorption of either low or high molecular weight solute (*i.e.*, "selective" absorption) is difficult to obtain, but it is thought that such effects were not occurring as (*1*) consistent osmotic pressures were observed independently of the order in which solutions of varying concentrations were measured, and (*2*) in an additional experiment, there was no change in the observed osmotic pressure when the osmometer was filled successively with two solutions of the same concentration (*i.e.*, π = 0.420 and 0.419 cm. chloroform, respectively). Both "selective" and "nonselective" adsorption could have influenced the other determinations carried out on this sample. Similarly, density corrections are often extremely important when high molecular weight substances are being examined, particularly when comparisons are made between results from different osmometers: although negligible in the case of the Zimm-Myerson instrument, they are considerable in the Fuoss-Mead type. For example, if Cleverdon's result of 222,500 is corrected on the basis of an average height of solution column of 20 cm., the value of $\bar{M}_n$ is 215,000. Bawn's results may require a correction of the same order.

Thus, although we have attempted to account for most of the possible sources of error, our result for $\bar{M}_n$ is 10% lower than Cleverdon's corrected

value and 20% higher than Hookway's value. Apart from the unpredictable effect of "selective" adsorption occurring in measurements using either denitrated collodion or polyvinyl alcohol membranes, the most likely reason for the discrepancy is the low molecular weight "tail" escaping notice in the measurements giving the higher values, as Hookway claims that polyvinyl alcohol membranes are semipermeable down to $\bar{M}_n \approx 2000$.[3] Low molecular material was certainly present in the sample as shown in our measurements, although it was not apparent in those of Cleverdon.[2] It appears likely that the nonideal semipermeability of the membranes could account for the discrepancy. If this is so, then extrapolations applied for solute permeation in our measurements must be incorrect. However, it is difficult to reconcile this with the observed π *vs.* time curves unless there is an instantaneous diffusion of very small material in the few minutes required to adjust the levels, etc., before measurements could be taken.[8] It may also be noted that Staverman[9] believes that this method of correcting for solute diffusion is inadequate.

Further investigations are clearly necessary, and probably comparisons should be made using these different membranes and osmometers on a sharply fractionated high molecular weight polymer.

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Physicochemical Studies on Starches. Part II. The Oxidation of Starches by Potassium Metaperiodate.*

By D. M. W. Anderson, C. T. Greenwood, and E. L. Hirst.

[Reprint Order No. 5721.]

The oxidation of 18 different starches by potassium metaperiodate has been studied. At constant temperature, the time taken for the theoretical uptake of periodate ion varies from starch to starch. Only traces of glucose were found in the hydrolysates from oxidised starches when oxidation was continued until over-consumption of periodate had occurred. Potentiometric-titration studies showed that, in the presence of all reaction products, formic acid is quantitatively determined by titration to pH 6·25. Oxidised starches are stable below this pH, but decompose rapidly in dilute alkali, yielding acidic products in quantity. Oxidised starches bind about 2% of the formic acid released during oxidation; hence complete estimation of this acid can only be made by titrating the *heterogeneous* reaction mixture to pH 6·25. The effect of the presence of protein on the periodate oxidation of oat-starch samples has been investigated. Values for the ratio of non-terminal to terminal groups of the starches studied are presented, the accuracy claimed being ±0·5 glucose unit. The average length of unit-chain of the corresponding amylopectin components has been calculated from these values and the results of determinations of the percentages of amylose.

In recent years there has been an increasing tendency for the methylation method of determining the ratio of non-terminal to terminal groups [*i.e.*, $(\bar{R})$] in starches to be superseded by the method involving the estimation of formic acid released from the non-reducing end-groups during periodate oxidation. The periodate method has the advantages of simplicity and speed and requires about ten times less material. Theoretically, it can also provide additional evidence for the presence or absence of inter-chain linkages involving $C_{(2)}$ or $C_{(3)}$ (Halsall, Hirst, Jones, and Roudier, *Nature*, 1947, **160**, 899) and, in conjunction with methylation data, of glucose residues linked solely through $C_{(1)}$ and $C_{(6)}$ (Brown, Halsall, Hirst, and Jones, *J.*, 1948, 27). However, the accuracy of periodate determinations of the value of $(\bar{R})$ for starches has been quoted as ±10% (*idem*, *loc. cit.*). There has also been considerable disagreement in the values obtained for the average lengths of unit-chain of amylopectins by different workers (see Table 1). The studies reported here were undertaken in an effort to reduce the experimental errors

Table 1. *Values quoted from periodate oxidation results for the average length of unit chain of amylopectins.*

	Reference			
Source of starch	A	B	C	D
Maize	20	—	25—26	25·2
Potato	24—26	—	27	—
Tapioca	20	22	23	26·6
Wheat	21	—	23	—

A, Brown, Halsall, Hirst and Jones, *loc. cit.*; B, Meyer and Settele, *Helv. Chim. Acta*, 1953, **36**, 197; C, Potter and Hassid, *J. Amer. Chem. Soc.*, 1948, **70**, 3488; D, *idem*, *ibid.*, 1951, **73**, 997.

involved in periodate oxidations, and so obtain accurate values of $(\bar{R})$ for use in conjunction with the results from differential potentiometric-titration studies of the iodine uptake of starches and their components.

* Part I, *J.*, 1954, 3769.

Oxidations were carried out with potassium metaperiodate (Halsall, Hirst, and Jones, *J.*, 1947, 1399) at 15—16° and at 20—21°. At both temperatures, the time required for the theoretical uptake of 1·03—1·05 moles of periodate per anhydroglucose unit varied for different starches. Some typical results are shown in Table 2. In no case was the theoretical uptake reached in less than 240 hr. at 15—16°, and even after 350 hr. at this

TABLE 2. *Periodate uptake, in moles per anhydroglucose unit.*

		Time of oxidation (hours)							
Source of starch	Temp.	72	120	160	200	240	280	336	432
Barley I [a]	15—16°	—	—	0·91	0·99	1·01	1·04	—	—
Oat [b]	,,	0·77	0·80	0·86	0·96	1·02	—	1·05	—
Potato I [c]	,,	0·81	0·86	0·89	0·95	0·98	1·00	1·02	—
Potato II [d]	,,	—	—	0·88	0·94	0·96	—	1·01	1·04
Potato III [e]	,,	—	—	0·88	0·94	0·97	—	1·03	1·03
Rice [c]	,,	—	—	0·72	0·84	0·93	1·01	1·03	—
Sweet potato [c] ...	,,	—	—	—	0·97	1·01	—	—	—
Waxy maize [c]	,,	—	—	0·91	0·92	0·96	1·00	1·03	1·05
Oat [b]	20—21	—	0·97	1·02	1·03	—	1·08	—	—
Potato II [d]	,,	—	0·97	1·03	1·04	1·09	1·13	—	—
Rice [c]	,,	—	0·95	1·01	1·03	1·06	1·09	—	—
Waxy maize [c]	,,	—	0·98	1·02	—	1·03	1·05	—	—

[a] McWilliam and Percival, *J.*, 1951, 2259; [b] Anderson and Greenwood, unpublished work; [c] Samples described in *J.*, 1948, 27; [d] *Var.* "Golden Wonder"; [e] *Var.* "Redskin" ([d,e] Greenwood, unpublished work).

temperature, very little over-consumption of periodate (and hence very little over-oxidation of starch) occurs. At 20—21°, the oxidation is about 30% faster, but there is a greater tendency for over-oxidation.

It follows that the time required for the periodate-uptake of a starch to reach the theoretical value *at constant temperature* must be determined. The amount of formic acid liberated in a shorter time cannot be quantitative. Although a longer time may even be necessary to allow for hydrolysis of intermediate complexes (Hughes and Nevell, *Trans. Faraday Soc.*, 1948, **44**, 941), it has been found that in the final stages of oxidation the release of formic acid follows consumption of periodate without apparent delay (see Fig. 1). The differing oxidation times required could be explained by differences in chain length and degree of multiple branching of the amylopectin fractions, by differences in natural granular structure, or by alteration in the physical characteristics of the granules arising from differing methods of preparation. As a result, it appears unlikely that the oxidation time for any simple saccharide can be taken as a standard for starch. In particular, it is difficult to justify the procedure of Morrison, Kuyper, and Orten (*J. Amer. Chem. Soc.*, 1953, **75**, 1502), who apply a fixed correction factor, based on the percentage of the theoretical formic acid released in the same time from sucrose; results agreeing with those of Halsall, Hirst, and Jones (*J.*, 1947, 1427) have been obtained here which show that release of formic acid from sucrose is abnormally slow and non-quantitative. There is no reason to suspect that starches differ from the model of methyl β-D-maltoside (Brown, Halsall, Hirst, and Jones, *loc. cit.*) which releases formic acid quantitatively in 150 hr. at 15°, in any respect other than having variably slower rates of oxidation.

After several starches had been oxidised for the time found necessary for the theoretical uptake of periodate, they were examined by the methods described by Hirst, Jones, and Roudier (*J.*, 1948, 1779) for the isolation and hydrolysis of the oxidation products, and for the determination of glucose in the hydrolysates. Quantities of glucose between 0·5 and 1% were found. When further samples of the same starches were oxidised for at least 100 hr. longer in each case, traces of glucose, too small for estimation, were detectable only by examination of the chromatograms under ultra-violet light. Recent papers have reported the presence of 1—2% of glucose in hydrolysates after oxidation at room temperature for 240 hr. (Hirst, Jones, and Roudier, *loc. cit.*) and for 150 hr. (McWilliam and Percival, *loc. cit.*), but these authors were undecided whether this was of structural significance or due to incomplete oxidation. The latter explanation is supported by the present work, which shows that about 1% of glucose residues in the starches examined are

abnormally but not completely resistant to periodate attack. This suggests that 1 : 2- or 1 : 3-glucosidic linkages are not present, and is in agreement with the work of Gibbons and Boissonnas (*Helv. Chim. Acta*, 1950, **33**, 1477). However, the polyaldehydic oxidation products are very easily hydrolysed, giving solutions containing a brown precipitate, and Jackson and Hudson (*J. Amer. Chem. Soc.*, 1938, **60**, 989) reported that during hydrolysis some destruction of material occurred and polymer degradation was incomplete. For this reason, Abdel-Akher, Hamilton, Montgomery, and Smith (*ibid.*, 1952, **74**, 4970) hydrolysed the corresponding polyalcohols and claimed that 1 : 3-linkages exist since 1% of glucose residues was found. However, the period of oxidation used may not have been sufficient for complete oxidation.

The present work has shown that aqueous suspensions of the polyaldehydes obtained are stable in the range pH 3—6·25, but decompose readily in 0·01—0·001N-alkali, releasing acidic products even in a nitrogen atmosphere, with decomposition ceasing when the pH

FIG. 1. FIG. 2.

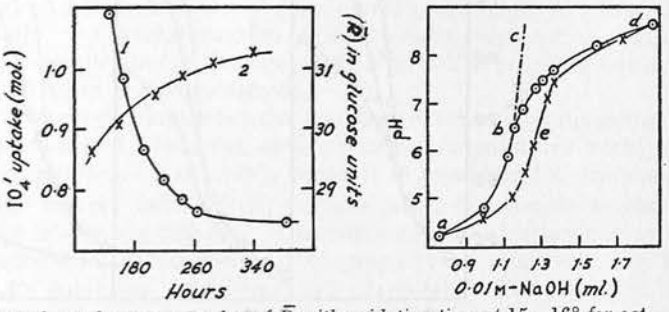

FIG. 1. *Variation of periodate-uptake and apparent value of* $\bar{R}$ *with oxidation time at* 15—16° *for oat starch.*

Curve 1. $(\bar{R})$ *(glucose units).*
Curve 2. IO_4'*-uptake (moles/anhydroglucose unit).*

FIG. 2. *Titration curves for a reaction mixture.*

Curve abc. Control $H{\cdot}CO_2H$–NaOH.
Curve abd. Reaction mixture–NaOH.
Curve dea. Back-titration curve for mixture–$H{\cdot}CO_2H$.

has dropped to 6·25. For example, immediately after potentiometric titration of a reaction mixture (Fig. 2, curve *abd*, of which part *bd* is time-dependent), the excess of alkali was back-titrated with standard formic acid (curve *dea*); 0·16 ml. of acid was found to have been liberated in the time taken (about 15 min.) to titrate from *b via d* to *e*. Liberation of acid in this manner may explain some anomalous results which have been reported involving over-production of acid during periodate oxidations in alkaline-buffered systems. Similarly, when a calculated excess of potassium hydroxide was added to a series of periodate oxidations of waxy maize starch at 15—16°, it was found that, although the rate of periodate uptake was normal (0·55 mole/162 g. of starch after 40 hr.), the acid liberated reached the theoretical value in only 38 hr. and continued to increase. The alkali-sensitivity of some aldehydes obtained by periodate oxidation has been investigated by Head (*J. Text. Inst.*, 1947, **38**, T 389), and it is considered that the acid formation reported here is due to alkaline hydrolysis of the acetal linkages accompanied by continued conversion of $-CHO$ into $-CO_2H$ by Cannizzaro-type reactions and *not* to over-oxidation by periodate, as the uptake did not exceed 1·1 moles/anhydroglucose unit (cf. Barry, *J.*, 1942, 578).

There was the possibility that esterification of $C_{(6)}$ in the starch may occur with some of the liberated formic acid (cf. Gottlieb, Caldwell, and Hixon, *J. Amer. Chem. Soc.*, 1940, **62**, 3342; Tarkov and Stamm, *J. Phys. Chem.*, 1952, **56**, 262). This would account for the release of some acid when the oxidation product is treated with alkali, and would imply that the estimation of formic acid by titration of aliquot portions centrifuged free from oxidised starch granules would not be quantitative. As esterification might have already occurred during preparation of the polyaldehydes, quantities were shaken for twelve days

at 15—16° with concentrations of formic acid ten times greater than that normally released during oxidation, so that further esterification could occur. The formic acid was recovered quantitatively: in a similar experiment with pure starches, evidence of 0·2% removal of acid was obtained. Hence no significant quantity of formic acid becomes chemically bound as ester during periodate oxidations lasting 12 days at 15—16°.

As Halsall, Hirst, Jones, and Sansome (*Biochem. J.*, 1948, **43**, 70) have obtained evidence that different samples of the same starch, derived from plants differing in botanical variety and growth-conditions, contain the same proportions of end-group, and as this result has been substantiated (see Table 3), the deviating periodate values in

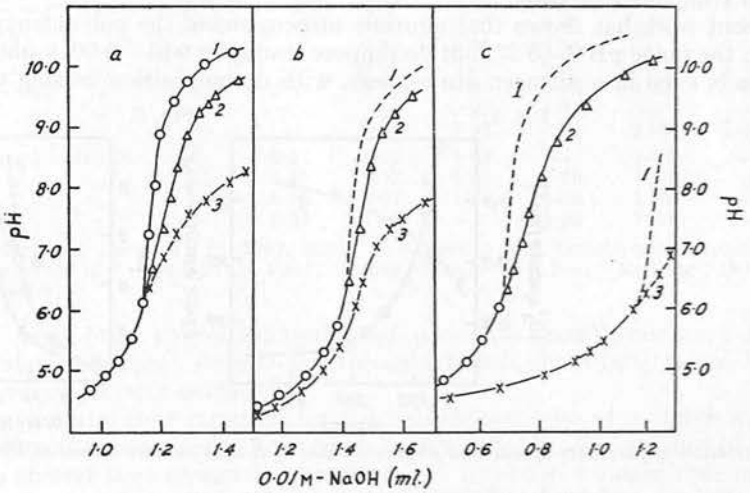

FIG. 3*a*. *Titration curves for control solutions.*
Curve 1. $H{\cdot}CO_2H$–NaOH.
Curve 2. ($H{\cdot}CO_2H$ + KCl + $NaIO_4$ + *ethylene glycol*)–NaOH.
Curve 3. [*As* (2) + *oxidised starch*]–NaOH.

FIG. 3*b*. *Titration curves for reaction mixtures.*
Curve 1. *Control* $H{\cdot}CO_2H$–NaOH.
Curve 2. *Centrifuged reaction mixture*–NaOH.
Curve 3. *Uncentrifuged reaction mixture*–NaOH.

FIG. 3*c*. *Titration curves for a proteinaceous reaction mixture.*
Curve 1. *Control* $H{\cdot}CO_2H$–NaOH.
Curve 2. *Centrifuged reaction mixture*–NaOH.
Curve 3. *Uncentrifuged reaction mixture*–NaOH.

Table 1 could possibly be explained by experimental errors arising through incomplete oxidation and the use of differing procedures for the determination of formic acid. Abdel-Akher and F. Smith (*J. Amer. Chem. Soc.*, 1951, **73**, 994) prefer the iodometric method; Kerr and Cleveland (*ibid.*, 1952, **74**, 4036) titrate to pH 7·1, and papers in which titration to pH 5·5, 6·0, 8·0, and 8·2 was used have been referred to by Morrison, Kuyper, and Orten (*loc. cit.*), who showed that choice of end-point in the range pH 5·5—8·0 should have little influence on quantitative titration of *pure* formic acid. This is correct, but potentiometric titration studies carried out here, supported by iodometric determinations, have shown that quantitative titration of formic acid in the presence of all normal oxidation products is complete at pH 6·25, and that over-titration to pH 8·0 would reduce the value obtained for a true ($\bar{R}$) value of 25—30 by about 20%. In Fig. 3*a*, which shows typical potentiometric titration results for control solutions, the curve for pure formic acid (curve 1) differs above pH 6·5 from that for the same quantity of acid in the equivalent of a centrifuged reaction mixture (curve 2). A still greater divergence occurs above pH 6·25 when 50 mg. of oxidised starch (carefully washed free from acid) are added, so giving the equivalent of an uncentrifuged reaction mixture (curve 3). Cori and Larner (*J. Biol. Chem.*, 1951, **188**,

17) noted " an apparent buffering action " during titration of periodate reaction mixtures : this is now explained by the alkaline degradation of oxidised starch. From the equivalence point of curve 1 at pH 7·1, it is seen that under these experimental conditions, titration of formic acid is complete by pH 6·5 for curve 2 and pH 6·25 for curve 3. The divergence of these curves above pH 6·25 is due to inclusion in the titration of substances other than formic acid.

Fig. 3 *b* shows the titration curves for a typical reaction mixture, curve 2 being for 10 ml. of centrifuged solution and curve 3 for 10 ml. of the heterogeneous mixture. Although both curves are superimposable with curve 1 for pure formic acid to pH 6·25, they are not themselves coincident over any range, and the end-points at pH 6·5 for (2) and pH 6·25 for 3 differ by 0·03 ml. Differences of this order (about 2% of the total titration) were consistently found. Investigation showed that the formic acid in the reaction mixture is not uniformly distributed, about 2% being loosely bound to the polyaldehydic oxidation product and removable by several washings with distilled water. Hence removal of the starch oxidation product before titration of formic acid gives values of $(\bar{R})$ which are about 0·5 glucose unit high. By careful titration of the heterogeneous reaction mixture to pH 6·25, any bound acid is included in the titre and there is no risk of including any acid arising from alkaline degradation of the polyaldehyde.

The behaviour of some protein-contaminated oat starches [obtained as intermediates in the purification of the pure starch (Anderson and Greenwood, unpublished work)] on periodate oxidation has been studied. For protein contents of less than 3%, uptake of periodate is normal, and the correct value of $(\bar{R})$ is given when the sample weight is corrected for the percentage of protein present. The differences in titration curves of centrifuged and non-centrifuged samples increase with increasing protein-content. Fig. 3 *c* shows the curves obtained for a product containing 23% of protein. The end-point for the centrifuged solution (pH 6·5; curve 2) gives a value of $(\bar{R})$ of 40, whilst that for the non-centrifuged solution (pH 6·25; curve 3) gives the correct value of $(\bar{R})$ of 28. Thus

TABLE 3. *Average values of* $(\bar{R})$ *found for unfractionated starches, and calculated number of glucose residues per non-reducing end-group in the amylopectin fraction.*

Source of starch	Temp.	No. of determns.	Oxidation time (hr.) *	Av. value of $(\bar{R})$ †	Amylose ‡ content (%)	Calc. chain-length for amylopectin
Arrowroot [e]	15—16°	3	284—312	27·3	20·5	21·7
Banana [e]	15—16	2	244—336	26·3	16·8	21·9
Barley I [a]	15—16	4	260—282	29·5	22·0	23·0
Barley II [f]	15—16	2	260—282			
Iris germanica [g]	15—16	2	262—308	28·0	27·0	20·4
Maize [c]	15—16	2	300—384	26·5	24·0	20·1
Oat I and II [b]	15—16	5	240—318	27·4	26·0	20·3
	20—21	1	164—240			
Parsnip [h]	15—16	2	268—360	23·0	11·1	20·4
Pearl manioc [e]	15—16	2	244—312	24·1	15·7	20·3
Potato I [c]	15—16	2	291—383	28·3	20·4	22·5
Potato II [d]	15—16	2	336—455	28·3	21·0	22·4
	20—21	1	186			
Potato III [e]	15—16	2	335—455	28·3	21·0	22·4
Rice [c]	15—16	3	286—384	27·5	18·5	22·4
	20—21	1	164—212			
Sago [e]	15—16	2	244	25·0	26·0	18·5
Sweet potato [e]	15—16	2	266—310	28·2	17·8	23·2
Tapioca [e]	15—16	4	264—300	26·2	16·7	21·8
Waxy maize [e]	15—16	4	302—400	20·0	<1	20
	20—21	1	164—284			
Wheat [e]	15—16	4	260—306	26·2	25·0	19·6

* The time necessary (found by separate expt.) for periodate uptake to reach 1·03—1·05 moles/162 g. starch : the range quoted shows the period in which no over-oxidation occurred, the formic acid released being constant within the limits corresponding to $(\bar{R}) \pm 0{\cdot}5$ glucose unit.

† All values obtained were within $\pm 0{\cdot}5$ glucose unit from the average.

‡ Values obtained from potentiometric iodine titration curves (Anderson and Greenwood, unpublished work).

[a–e] See Table **2.** [f] Aspinall, Hirst, and McArthur. [g] Aspinall and Johnstone. [h] Greenwood (all unpublished work).

the presence of protein causes further complex-formation with formic acid, and the *heterogeneous* reaction mixture must be titrated. For samples containing more than 23% of protein, periodate uptake exceeds the theoretical value and no reliable estimate of $(\bar{R})$ can be obtained from the potentiometric-titration curves.

The potentiometric-titration method has been found to be the simplest and most reproducible for determining the formic acid released on periodate oxidation. Whilst the iodometric method, which determines total acidity, gives good agreement in determinations on pure starches, the potentiometric method has the advantage of showing from the shape of the titration curve whether acids other than formic are being titrated, so indicating the presence of impurity. The steam-distillation method has been found to give less consistent results for the small amounts of formic acid normally released, and it is slower.

The average values of $(\bar{R})$ found for the starches studied are presented in Table 3, the experimental error being within $\pm 0{\cdot}5$ glucose unit. The values deduced for the average length of unit-chain in the corresponding amylopectin components are also shown.

Under the experimental conditions described, periodate oxidation is a reliable and easy routine method for determining values of $(\bar{R})$ for starches, having an accuracy and reproducibility better than that of the methylation technique.

Experimental

All starch samples were dried *in vacuo* at 80° for several hours. Reagents were of analytical grade, or were purified as described by Halsall, Hirst, and Jones (*loc. cit.*). Nitrogen and sodium hydroxide used during potentiometric titrations were free from carbon dioxide.

Periodate Oxidations.—Starch (250—400 mg.) was suspended in 0·56M-potassium chloride (60 ml.) to which was added 0·2M-sodium metaperiodate (20 ml.). Within these limits the rate of oxidation was independent of the weight of starch. Reaction flasks were shaken continuously in the dark in a constant-temperature room.

Potentiometric Titrations.—Samples (10 ml.) were withdrawn by pipette at the required times. Ethylene glycol (1 ml.) was added and the mixture shaken in the dark for at least 10 min., the time found necessary for complete reaction between the glycol and the suspension of potassium periodate. (All excess of periodate *must be destroyed* before the start of a potentiometric titration.) Nitrogen was bubbled through the mixture for 10 min. before titration with 0·01M-sodium hydroxide (semimicro-burette). The passage of nitrogen was continued throughout the titration, which was followed by means of a glass electrode and Pye mains-operated pH-meter. Blank determinations showed that generally no correction was required for the acidity of the starch samples or of other reagents.

Withdrawal of samples by pipette whilst the reaction flask was *shaken gently* introduced no error. Each of seven 10-ml. portions withdrawn consecutively from a reaction mixture gave the same titre with sodium hydroxide, and, further, the value of $(\bar{R})$ deduced was the same as that obtained from titrations of the entire contents of each of three individual reaction mixtures (10 ml. each) after the same oxidation time. This procedure did not, however, give consistent results for determinations of excess of periodate as the more rapid sedimentation of potassium metaperiodate made impossible the withdrawal of samples homogeneous with respect to this component.

It was shown that no loss of acid occurred when nitrogen was passed through 0·0015M-formic acid for 1½ hr.

Oxidation of Formic Acid by Potassium Metaperiodate.—No loss of formic acid or consumption of periodate occurred when 0·0015M-formic acid was shaken with a saturated solution of potassium metaperiodate for 28 days in the dark at 15—16°. At 20—21°, however, the concentrations of formic acid and of periodate decreased by 3% after 15 days, and by 6% after 21 days.

Distribution of Formic Acid in Reaction Mixtures.—After oxidation for 240 hr., a starch–potassium periodate reaction mixture (80 ml.) was divided into two. The first half was centrifuged, and portions (10 ml.) of the clear supernatant liquid were titrated (after destruction of excess of periodate) against 0·01024M-sodium hydroxide to pH 6·25. The average titre was 1·40 ml. From the second half, two *heterogeneous* 10-ml. portions were withdrawn, treated with glycol, and titrated to pH 6·25: the average titre was 1·43 ml. The remaining two 10-ml. portions were treated separately with glycol, then centrifuged, and the oxidised granules were

washed three times with distilled water by centrifugation. The combined supernatant liquids and washings were then titrated to pH 6·25, the average titre being 1·43 ml.

Determination of Periodate Uptake.—Residual periodate concentration was determined by Fleury and Lange's indirect method (*J. Pharm. Chim.*, 1933, **17**, 107). This method gave satisfactory results in the presence of all reactants and products when the reaction mixture, to which had been added excess of bicarbonate, arsenite, and iodide, was *shaken in the dark for* 15 *min.* before back-titration with iodine. Periodate uptake was determined by analysis of a series of individual mixtures (10 ml., containing *ca.* 50 mg. of starch). The stoppers of the conical reaction flasks were lubricated with a little silicone grease.

Periodate Uptake and Formic Acid Release from Sucrose.—Results obtained (expressed in moles/mole of sucrose) were : (*a*) periodate uptake : 2·98 (262 hr.) ; 3·12 (300 hr.) ; (*b*) formic acid release : 0·87 (262 hr.) ; 0·88 (286 hr.) ; 0·89 (352 hr.) ; 0·91 (408 hr.) ; 0·92 (420 hr.) ; 0·93 (570 hr.).

Interaction of Formic Acid with Starches and their Oxidation Products.—The following mixtures were shaken for 240 hr. in the dark at 15—16° : (*a*) control solution of formic acid (10 ml.) ; (*b*) formic acid (10 ml.) and oat starch (64·35 mg.) ; (*c*) formic acid (10 ml.) and periodate-oxidised oat starch (65·18 mg.) ; (*d*) formic acid (10 ml.) and waxy maize starch (60·08 mg.) ; and (*e*) formic acid (10 ml.) and periodate-oxidised waxy maize starch (55·60 mg.). The contents of each reaction flask were then titrated to pH 6·25 against 0·00901M-sodium hydroxide, the titres obtained being (*a*) 16·15, (*b*) 16·05, (*c*) 16·15, (*d*) 15·85, and (*e*) 16·15 ml.

Examination of Polyaldehydic Oxidation Products.—No colour reaction occurred with (*a*) iodine–potassium iodide, (*b*) dilute sulphuric acid–potassium iodide, or (*c*) sulphuric acid–diphenylamine ; Fehling's solution and Schiff's reagent were reduced. The release of acidic decomposition products from the oxidised starch in the presence of alkali was shown in the following experiments : (*a*) Oxidised oat starch (50 mg.) was added to water (5 ml.) which had been boiled, and then cooled, in the presence of nitrogen. 0·01024M-Sodium hydroxide (1 ml.) was added, and the mixture shaken for 15 hr. The pH was then 6·25, and did not decrease further during 24 hr. Further addition of 0·01024M-sodium hydroxide (1 ml.) gave an initial value of pH 10·5, which decreased overnight to pH 6·25 and did not decrease further. (*b*) Oxidised waxy maize starch (35·92 mg.) was shaken with 0·00901M-sodium hydroxide (20 ml.) for 17 hr. Titration of the excess of alkali to pH 6·25 required 1·70 ml. of 0·0147M-formic acid. Hence 232 g. of oxidised starch would liberate 1 l. of N-acid (duplicate experiment gave 273 g. as the apparent neutralisation equivalent).

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Physicochemical Studies on Starches. Part III. The Interaction of Starches and Branched α-1 : 4-Glucosans with Iodine; and a Valve Microvoltmeter for Differential Potentiometric Titrations.*

By D. M. W. Anderson and C. T. Greenwood.

[Reprint Order No. 6182.]

The uptake of iodine by 20 different starches has been studied by differential potentiometric titration, and iodine affinities for these samples are quoted. Contaminating protein alters significantly the apparent iodine affinity of starches. The iodine binding power of amylopectins, glycogens, and other branched α-1 : 4-glucosans has been studied. The differences observed, in conjunction with data for average unit-chain and external-chain lengths, suggest that variations in fine structure (*i.e.*, in degree of multiple branching) exist. In addition, details are given of a valve microvoltmeter developed to extend the scope and accuracy of differential potentiometric iodine titrations.

A quantitative estimate of the amount of iodine bound by starch and its components can be obtained by the potentiometric-titration method introduced by Bates, French, and Rundle (*J. Amer. Chem. Soc.*, 1943, **65**, 142). Colorimetric methods developed subsequently (Hassid and McCready, *ibid.*, p. 1154; Bourne, Haworth, Macey, and Peat, *J.*, 1948, 924) are more arbitrary, although useful for comparative measurements. The optical absorption characteristics of the iodine complexes of different amyloses and amylopectins vary (cf. Baldwin, Bear, and Rundle, *J. Amer. Chem. Soc.*, 1944, **66**, 111; Kerr, Cleveland, and Katzbeck, *ibid.*, 1951, **73**, 3916), and the measurements are not absolute or capable of the same accuracy, particularly for amylopectin (cf. Higginbotham and Morrison, *Shirley Inst. Mem.*, 1948, **22**, 141).

Bates and his co-workers (*loc. cit.*) measured the potential between a bright platinum electrode in the starch–iodine–iodide solution and a standard calomel electrode, and thus were able to calculate the equilibrum concentration of free iodine in the mixture. However, the elegant *differential* method of Gilbert and Marriott (*Trans. Faraday Soc.*, 1948, **44**, 84) is much more satisfactory for accurate work at the necessary low free-iodine concentrations. In this technique, the starch–iodine–iodide solution and the blank–iodine–iodide solution are arranged as opposing half-cells connected by a salt bridge; the equilibrium free-iodine concentration in the starch solution can then be found directly, and separate titrations for reagent blanks are not required. As has recently been mentioned (Mould, *Biochem. J.*, 1954, **58**, 593), the scope of the differential technique and the accuracy and reproducibility of results obtained by its use depend on the availability of a null-potential indicator of great zero stability combined with high sensitivity. Such an electronic device, providing high sensitivity (30 mm./mv) and zero stability has already been described (Anderson and Greenwood, *Chem. and Ind.*, 1953, 476). This electrometer gives excellent results for routine analyses of unfractionated starches, but reliable readings are not possible when the free-iodine concentrations exceed 8×10^{-6}M. The logarithmic decrease in possible sensitivity with increasing free-iodine concentration in both half-cells is an inherent disadvantage of the differential-titration technique. Nevertheless, a ten-fold increase in sensitivity was sought, to permit an accurate study of the iodine-binding power of branched α-1 : 4-glucosans (*e.g.*, the amylopectin component of starch) on which relatively little work has yet been carried out. It appeared possible that such a study could give some details of fine structure.

* Part II, *J.*, 1955, 225.

Mikus, Hixon, and Rundle (*J. Amer. Chem. Soc.*, 1946, **68**, 1115) consider that the low iodine-binding power of branched α-1 : 4-glucosans is inexplicable in terms of hydrogen bonding (cf. Whistler and Hilbert, *ibid.*, 1945, **67**, 1161). They suggested that the large number of branch-points prevents helix formation and decreases the dipolar forces thought to be responsible for complex formation between iodine and the amylose component of starch. [Higginbotham (*Shirley Inst. Mem.*, 1949, **23**, 171) has suggested that, in amylopectin, adsorption of I_2 and I_3^- occurs in addition to complex formation in helices.] The amount of helix formation possible, and hence the iodine uptake, must be related to fine structure. The several model structures proposed for amylopectin [*i.e.*, the " laminated " structure (Haworth, Hirst, and Isherwood, *J.*, 1937, 577; Halsall, Hirst, and Jones, *J.*, 1949, 3200), the " herring-bone " structure (Staudinger and Eilers, *Annalen*, 1937, **527**, 195), and the " ramified " structure (Meyer and Bernfeld, *Helv. Chim. Acta*, 1940, **23**, 857)] all contain different arrangements of the same linear basic chains (Myrbäck and Sillén, *Acta Chem. Scand.*, 1949, **3**, 190), which Peat, Whelan, and Thomas (*J.*, 1952, 4546) have suggested be termed A-, B-, and C-chains. The three models differ, therefore, only in their ratio of A : B chains, *i.e.*, in the *degree of multiple branching*. Similar considerations also apply to other branched α-1 : 4-glucosans. Variations in fine structure must exist to explain the difference in limiting viscosity numbers of the two branched glucosans, amylopectin and glycogen (cf. Greenwood and Robertson, *J.*, 1954, 3769). Any method which can give further indications of differences in fine structure is important.

Experimental

Details of Valve Microvoltmeter.—The few valve millivoltmeters described in recent years (see, *e.g.*, Morton, *Trans. Faraday Soc.*, 1948, **44**, 588; Gray, *Discuss. Faraday Soc.*, 1950, **8**, 331, and personal communication; Scroggie, *Wireless World*, 1952, 14; Furman, *Analyt. Chem.*, 1954, **26**, 84) were found either to be incapable of modification for our purpose, or, when constructed, had a zero-drift about 100 times greater than required.

Attempts to improve the sensitivity of the original circuit (Anderson and Greenwood, *loc. cit.*) by using miniature valves of high mutual conductance ($g_m = 10$) with 22-v heaters (run from the stabilized high-tension supply) were unsuccessful as the valves would not function under these " under-run " conditions. The desired standard was finally achieved by improving the stability of both high- and low-tension voltage supplies, then amplifying the output by a matched pair of valves functioning as a cathode-coupled amplifier. This design had the advantage of retaining the satisfactory high input impedance and low grid current of the original circuit, and moreover was still simple, depending on fundamental balance of valves and components rather than compensating, and therefore complicating, circuitry. The final circuit is shown in Fig. 1*b*. Very accurate readings can be made up to free-iodine concentrations of 10^{-5}M.

Fig. 1*a* shows how a harmonic-filtered constant-voltage transformer supplies the input voltage to an Ediswan stabilized power unit (Type R1095) and to an accumulator trickle-charger. The latter charges a pile of 12 × 2-v cells (arranged in series/parallel to give 4-v and 6-v outputs) at the same currents as are being taken by the two pairs of valve filaments. A highly insulated switch allows the galvanometer (sensitivity 109 mm./microamp., internal resistance 402 ohm) to be connected either between K_1 and K_2 (so giving the original circuit; sensitivity = 30 mm./mv) or between A_3 and A_4, which gives a sensitivity of 315 mm./mv. In conjunction with the low rate of zero drift attained, this permits potentials of 1 microvolt to be measured. The circuit is extremely stable towards external electrical interferences, since both pairs of valves have been selected *under actual operating conditions* as the best matched pairs obtainable from a large number. The operating conditions of both pairs differ, and are to some extent interdependent; the choice of individual valves to form pairs and of optimum values for the resistors could only be made by continued " refinements." Wire-wound resistors, matched in pairs to within 1%, are used throughout, and all grid leads are of screened-type coaxial cable. For best results, the electrometer must be adequately protected against vibration, mechanical shock, and local changes in room temperature. The value of R_6 giving the optimum ratio of sensitivity to stability is 330 ohms; decreasing this value gives increased sensitivity, but the circuit may then tend to oscillate, creating instability. Although this can be minimized by inserting " grid-stopper " resistors (47,000 ohms; 0·5 w) inside the grid top-caps of V_3 and V_4, the thermal effects associated with these resistors contributed to zero drift. The introduction of negative feed-back, either by connecting V_3A to V_1G, and V_4A to V_2G, *via* 1 megohm

resistors, or by cross-connecting V_4A to V_3Sc and V_3A to V_4Sc, reduced rather than improved stability, and it was shown that zero drift is largely due to fluctuations in the low-tension, and not in the high-tension, supply. Absolute matching of V_3 and V_4 is achieved by connecting both V_3 and V_4 grids to V_1K (with V_1G to V_1K *via* a 2-megohm resistor), and, with P_4 pre-set at its optimum value, P_3 is adjusted so that the galvanometer deflection when connected across A_3/A_4 is zero. After V_4G has been returned to V_2K, P_3 is never altered, and all zeroing adjustments are made by using the "set zero" coarse and fine controls for both A_3/A_4 and K_1/K_2 systems.

The two-way switch shown in the input circuit to V_1 (Fig. 1*b*) must be very highly insulated and must make-before-break so that the grid of V_1 is never on open circuit. A satisfactory switch was made from a thick block of paraffin wax containing pools of mercury, between which contact was made by a tilting copper-wire framework. The inter-electrode resistance is

FIG. 1(*a*) *and* (*b*).

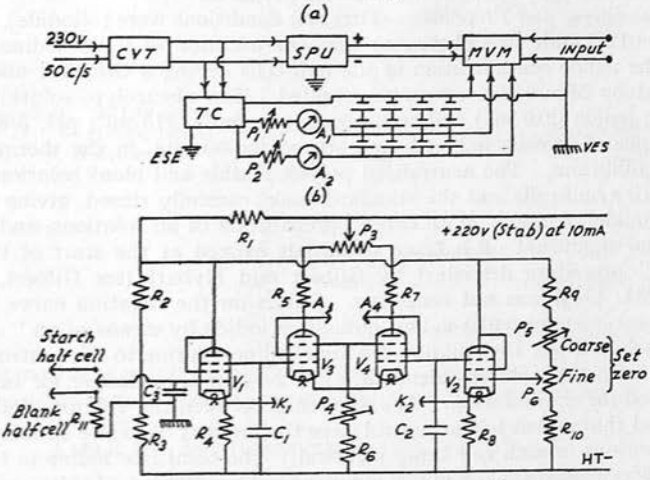

FIG. 1(*a*). *Block diagram of circuit.*

CVT, Constant voltage transformer. SPU, Stabilized power unit. MVM, Microvoltmeter. TC, Trickle-charger. ESE, Electricity supply earth. VES, Virtual earth system. A_1, *Ammeter (set at 0·88 amp.).* A_2, *Ammeter (set at 1·25 amp.).* P_1, 15 Ω/5 *amp.* P_2, 12 Ω/7 *amp.*

FIG. 1(*b*). *Microvoltmeter circuit.*

C_1, C_2, 0·01 μF, *mica.* C_3, 0·1 μF, *mica.* P_3, 500 Ω; P_4, 250 Ω; P_5, 10 *K* (*all* 5 w). P_6, 10Ω/2 w. (*All linear, wire-wound.*)

R_1, 100 *K*; R_2, 68 *K*; R_3, R_{12}, 500 Ω; R_4, R_8, 10 *K*; R_5, R_7, 22 *K*; R_6, 250 Ω; R_9, 65 *K*. (*All* 5 w, *wire-wound.*)

R_{11}, 2*M*, 1 w, *carbon.*

V_1, V_2 = VR 116; V_3, V_4 = VR 65 (SP61); V_1 *and* V_2, *heaters* 4 v *at* 0·88 *amp.*; V_A, 50 v; V_G, 0 v, V_K, 1 v; V_3 *and* V_4, *heaters* 6 v *at* 1·25 *amp.*; V_A, 110 v; V_G, 1 v; V_K, 2·4 v.

approximately 2 megohms; when the zero-reading of the electrometer is being checked, the grid of V_1 is therefore returned to earth *via* R_{11} so that the operating conditions of V_1 are changed as little as possible.

Details of Titration Cells.—The titration cells (1-l. Pyrex flasks) and salt bridge were similar to Gilbert and Marriott's (*loc. cit.*), except that stirring was automatic and continuous. Additions of iodine were made *via* additional necks in each flask. All four necks were fitted with ground-glass joints, enabling the apparatus to be completely sealed, stirring being made through Quickfit stirrer glands. [No loss of iodine occurred through volatilization in the time taken for titration (*i.e.*, 40 min.).] The electrodes consisted of platinum foil (2 × 2 cm.). By careful strain-free construction and thorough cleaning, it was possible to obtain a pair of electrodes between which no potential difference existed when placed in the same solution of electrolyte.

Reagents.—All reagents were of "AnalaR" grade, used without further purification.

Preparation of Starch Solutions.—All samples were exhaustively extracted with boiling methanol to remove traces of fat. This is essential. For example, an undefatted sample of commercial rice starch apparently contained 11·8% of amylose, but after being refluxed

with methanol (5 treatments; each of 3 hr.) a constant value of 18·5% of amylose was given (7·3% of the original weight of starch was extracted by the methanol). After removal of fat, samples were dried *in vacuo* at 80° for several hours before being weighed by means of a stoppered weighing-stick into a graduated flask. Suitable weights for titration were: starch, 10 mg.; amylopectin and glycogen, 30 mg. Dissolution was achieved by shaking the starch overnight at room temperature with 0·2M-potassium hydroxide (10 ml.) after moistening it with ethanol (2 drops). In certain cases it was necessary to heat the mixture at 95° for 3 min. before shaking. [The effect of pretreatment of whole starch with alkali was investigated as the amylose component degrades in this solvent (Bottle, Gilbert, Greenwood, and Saad, *Chem. and Ind.*, 1953, 541). Ageing at room temperature had no effect, and heating a starch in 0·2M- and 1M-potassium hydroxide for 30 min. at 95° had a negligible effect on the iodine affinity of the sample.] Immediately before addition to the titration half-cell, the alkaline polysaccharide solutions were brought to pH 5·85 by the addition of a predetermined volume of 0·4N-phosphoric acid. A blank solution containing no starch was similarly prepared.

Titration Conditions and Procedure.—Titration conditions were: [iodide], 0·01M; pH, 5·85; temp., 20°. 0·01M-Iodide was chosen so that the addition of 0·01M-iodine–potassium iodide did not alter the iodide concentration in the half-cells during a titration, and thus corrections such as applied by Mould (*loc. cit.*) were avoided. The electrolyte solution (2 l.) contained 0·1M-potassium iodide (210 ml.) and M/15-phosphate buffer (15 ml.; pH, 5·85). This solution (800 ml.) was placed in each half-cell, and stirred for 30 min. in the thermostat to allow for temperature equilibrium. The neutralized polysaccharide and blank solutions were then added to their respective half-cells and the standard flasks carefully rinsed, giving a total volume of 840 ml. (*i.e.*, [iodide], 0·01M). With careful preparation of all solutions, and with temperature equilibration, no significant off-balance potentials existed at the start of titrations, and the "depolarizing" procedure described by Gilbert and Hybart (see Gilbert, Greenwood, and Hybart, *J.*, 1954, 4454) was not necessary. Points on the titration curve were obtained by adding small increments of 0·01M-iodine–potassium iodide by means of an "Agla" micrometer syringe to the solution cell, then adding the same iodine solution to the control until the concentration of free iodine in each was identical, after 2—5 min. (or longer for branched glucosans) had been allowed for equilibration. The difference between the volume of iodine added to the solution cell and that added to the control gave the amount of iodine bound by the starch, the iodide concentrations in each cell being identical. The total free iodine in the starch solution (*i.e.*, $I_2 + I_3^-$) was plotted against mg. of iodine bound per 100 mg. of polysaccharide.

Reproducibility of Technique.—Results were independent of the time taken to complete a titration curve (provided true equilibration had been achieved at each free iodine concentration), and also of the sample weight. The reproducibility is within $\pm 2\%$ of the iodine affinity for an unfractionated starch (*i.e.*, for a starch having an iodine affinity of 5·0%, the results of 6 determinations lay between 4·9 and 5·1%).

As described by Gilbert and Hybart (*loc. cit.*), addition of excess of thiosulphate enabled the titration curve for any starch sample to be repeated. When the titration was repeated at 24- or 48-hr. intervals for 14 days, the starch solution being left in contact with iodine throughout, the observed changes in iodine affinity could be attributed to retrogradation of the amylose component. For waxy maize starch, the titration curve was unaltered after the sample had been in contact with iodine for 17 days. Similarly, for rabbit-liver glycogen, the curve was unaltered after contact with iodine for 21 and 31 days.

Results and Discussion

Fig. 2 shows some typical titration curves for starch samples obtained by plotting the amount of bound iodine against the total free-iodine concentration. Each starch was characterized by its "iodine affinity," which is a measure of the preferential uptake of iodine by the linear amylose component. At the free-iodine concentration saturating the amylose, the amount of iodine bound by the amylopectin is not negligible (see Anderson and Greenwood, *Chem. and Ind.*, 1954, 642, and below). An estimate of the amount of iodine bound by the amylose component was therefore obtained by extrapolating the linear portion of the titration curve to zero free-iodine concentration. All iodine affinities quoted here were calculated on this basis. The percentage of amylose in a starch can be calculated from this value when the corresponding value for pure amylose is known (Bates and his co-workers, *loc. cit.*). However, as previously mentioned (Greenwood and Robert-

son, *loc. cit.*), the only accurate method of doing this involves experimental determination of the maximum iodine-binding power of the *pure* amylose component of the starch under examination. Hence, when it is not desired to fractionate any starch exhaustively, it is more satisfactory to quote its iodine affinity, rather than an arbitrary percentage of amylose (cf. Schoch in Radley, " Starch and its Derivatives," Chapman and Hall, London, 1953, Vol. 1, p. 123). For comparison with other workers' results, however, percentages of amylose have been calculated, using 19·2% of bound iodine as the maximum iodine-binding power of pure amylose under our experimental conditions (Greenwood and Robertson, *loc. cit.*).

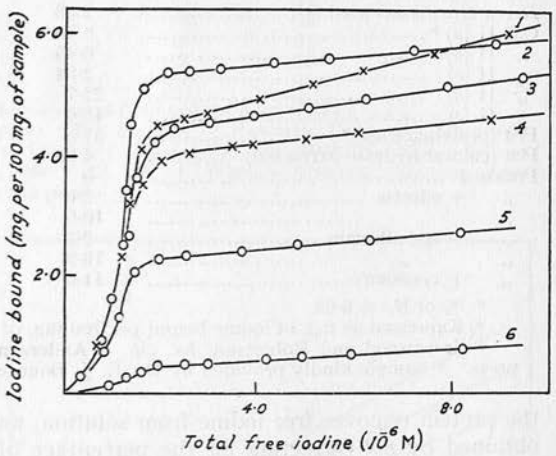

FIG. 2. *Typical iodine-titration curves for starches.*

1, *Potato* (*Golden Wonder*). 2, Iris germanica. 3, *Barley II*. 4, Hevea brasiliensis. 5, *Parsnip*. 6, *Waxy maize*.

Table 1 summarizes the results for starch samples. It is of interest that starches from different varieties of the same botanical source may show variation in the apparent percentage of amylose; this is in agreement with Doremur, Creshaw, and Thurber's results (*Cereal Chem.*, 1951, **28**, 308). The slope of the linear portion of the titration curve for all the potato starches studied was considerably greater than for other starches.

TABLE 1. *The iodine-binding power of starches.*

Source of starch *	No. of detns.	Iodine affinity †	Amylose (%) ‡	Slope of linear portion of curve §
Arrowroot	4	3·94	20·5	0·13
Banana	3	3·23	16·8	0·10
Barley I	2	4·22	22·0	0·12
Barley II	4	4·22	22·0	0·12
Hevea brasiliensis seed	3	3·86	20·0	0·10
Iris germanica	3	5·18	27·0	0·09
Maize	3	4·61	24·0	0·11
Oat I	2	5·00	26·0	0·13
Oat II	7	5·00	26·0	0·13
Parsnip	4	2·13	11·1	0·08
Pearl manioc	2	3·02	15·7	0·07
Potato I	7	3·94	20·4	0·28
Potato II	4	4·03	21·0	0·24
Potato III	2	4·03	21·0	0·23
Rice	6	3·55	18·5	0·09
Sago	4	5·00	26·0	0·08
Sweet potato	2	3·42	17·8	0·27
Tapioca	4	3·21	16·7	0·07
Waxy maize	5	0·27	1·4	0·06
Wheat	6	4·80	25·0	0·05

* Origin of samples as in Part II of this series (*loc. cit.*), except for *Hevea brasiliensis* seed (Greenwood and Robertson, *loc. cit.*).

† Expressed as mg. of iodine bound per 100 mg. of starch.

‡ Calc. as iodine affinity ÷ 19·2.

§ Expressed as % of iodine bound per total free-iodine concn. × 10^{6}(M) [range of total free iodine (2—10) × 10^{-6}M].

Contaminants affect the amount of iodine bound by a starch. Interference by fatty

acids is well known (cf. Mikus, Hixon, and Rundle, *loc. cit.*). In addition, protein has now been found to have considerable effect (see Table 2), and its presence causes distortion of the titration curve. For oat starch, the effect is to increase the apparent binding power;

TABLE 2. *The effect of contaminating protein on the iodine-binding power of starches.*

Starch sample	Protein (%) *	No. of detns.	Iodine affinity † Sample wt. uncorr.	Iodine affinity † Sample wt. corr. for protein
Hevea brasiliensis seed A [a]	0·31	3	—	3·86
Hevea brasiliensis seed B	2·56	2	—	3·01
Oat II (*a*) [b]	0·19	6	5·00	5·00
,, II (*b*)	0·45	3	5·44	5·48
,, II (*c*)	2·94	3	5·97	6·14
,, II (*d*)	22·7	3	5·27	6·80
,, II (*e*)	45·6	2	4·78	8·80
Pea (proteinaceous) [c]	37·5	2	—	9·20
Pea (chloral hydrate extracted)	4·93	1	—	15·1
Potato I	0	7	—	3·94
,, + edestin	2·0	1	—	3·78
,, ,,	10·0	1	—	3·05
,, + egg albumin	2·5	1	—	3·66
,, ,,	16·5	1	—	2·93
,, + tyrosine	11·0	1	—	3·45

* % of N, × 6·25.
† Expressed as mg. of iodine bound per 100 mg. of starch.
[a] Greenwood and Robertson, *loc. cit.* [b] Anderson and Greenwood, *J. Sci. Food Agric.*, in the press. [c] Sample kindly provided by Dr. E. J. Bourne; see *Nature*, 1948, **161**, 206.

the protein removes free iodine from solution, and estimates of the true affinity are best obtained by *not* correcting for the percentage of protein present. However, for rubber seed and pea starches, and for synthetic mixtures of protein with potato starch, the protein apparently suppresses starch–iodine complex formation. Interference by protein has also been found during the study of protozoal starches (unpublished observations). It is therefore essential to remove contaminating protein before titrations are attempted.

As briefly reported (Anderson and Greenwood, *loc. cit.*), the difference in iodine-binding power of normal amylopectins and glycogens is sufficiently large to characterize these two structure types. The iodine-binding power of these materials must be fundamentally related to differences in the average length of unit chain, degree of multiple branching, and external-chain length. For a group of polysaccharides having a similar degree of branching, it is also probable that variations in the amount of iodine bound are related to the length of *external chain* available for helix formation.

Titrations of different amylopectin samples have always shown evidence of preferential uptake of iodine by linear material. To compare iodine-binding powers, therefore, such preferential uptake has been corrected for by extrapolating the titration curve to zero free-iodine concentration, with this extrapolated point being taken as the origin for the iodine-binding curve. [This preferential uptake was presumably due to contaminating amylose; this is extremely difficult to remove (cf. Gilbert, Greenwood, and Hybart, *loc. cit.*), and the presence of some long branches in the amylopectin cannot be entirely excluded (cf. Swanson, *J. Biol. Chem.*, 1948, **172**, 825).] In all the glycogen samples so far examined there was no evidence of preferential uptake.

Fig. 3 and Table 3 show the results for some amylopectins and glycogens. In the range of concentrations employed, the amount of iodine bound is directly proportional, within experimental error, to the total free-iodine concentration. This would be expected if the iodine is bound as a co-linear core of iodine and tri-iodide molecules arranged end-to-end in the available helices. One iodine molecule can be accommodated in a helix of about six glucose units (Baldwin, Bear, and Rundle, *J. Amer. Chem. Soc.*, 1944, **66**, 111). Since the length of external chain available for helix formation is only 14—18 glucose units (*i.e.*, about three helices) for amylopectins, and 8—11 glucose residues (*i.e.*, 1—2 helices) for glycogen (cf. Manners, *loc. cit.*), the amount of iodine-binding possible is small.

Higginbotham (*loc. cit.*) has suggested that *adsorption* of iodine molecules (or tri-iodide

TABLE 3. *The iodine-binding power of branched α-1 : 4-glucosans.*

Sample	Linear material (%)	Slope of titration curve *	Av. length of unit chain	Length of external chain †
Barley II amylopectin	2·6	0·090	23 [a]	16 [g]
Hevea brasiliensis amylopectin	0·8	0·074	23 [a]	—
Oat I amylopectin	3·2	0·052	20·3 [a]	—
Ascaris lumbricoides glycogen	—	0·009	12 [b]	—
Rabbit-liver glycogen	—	0·006	13 [b]	8 [b]
Tetrahymena pyriformis polysaccharide	—	0·007	13 [b]	8—9 [b]
Waxy maize starch	1·4	0·060	20 [a]	15—16 [b]
Rabbit-liver glycogen	—	0·028	18 [c]	12
Wrinkled-pea amylopectin	3·4	0·485	36 [d]	—
Zea mays polysaccharide (insoluble)	0·26	0·019	12 [e]	8
,, ,, ,,	0·6	0·017	13 [f]	9
,, ,, (soluble)	0·6	0·007	11	7

* Expressed as for Table 2. † No. of glucose units removed on β-amylolysis +2·5.
[a] Part II, *loc. cit.*; [b] Manners, *Ann. Reports*, 1953, **50**, 288; [c] Haworth, Hirst, and Isherwood, *J.*, 1937, 377; [d] Potter, Silveira, McCready, and Owens, *J. Amer. Chem. Soc.*, 1953, **75**, 1335; [e] Dvonch and Whistler, *J. Biol. Chem.*, 1949, **181**, 889; [f] Dr. W. J. Whelan, personal communication; [g] Aspinall, Hirst, and McArthur, *J.*, 1955, in the press.

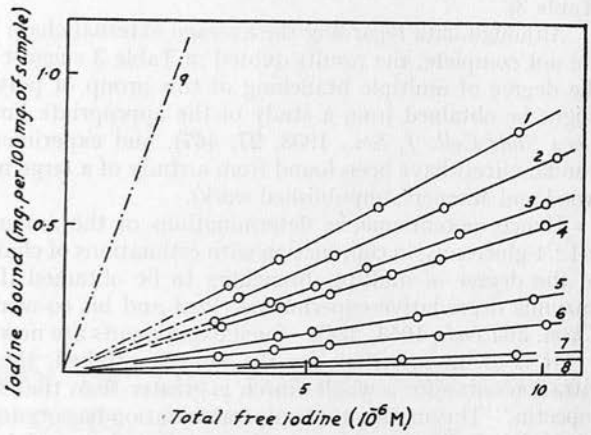

FIG. 3. *The iodine-binding power of branched α-1 : 4-glucosans.*

1, *Barley II amylopectin.* 2, Hevea brasiliensis *seed amylopectin.* 3, *Waxy maize starch.* 4, *Oat I amylopectin.* 5, *Glycogen (chain-length 18 glucose units).* 6, *Zea mays polysaccharides.* 7, Tetrahymena pyriformis *polysaccharide.* 8, *Rabbit-liver glycogen.* 9, *Wrinkled-pea amylopectin.*

ions) may also occur. However, at the low iodine concentrations used here, adsorption effects would be small, and are indeed unlikely to occur in view of the negligible effect on the titration curves of increased polysaccharide concentrations.

Fig. 3 shows that waxy maize starch behaves as a typical amylopectin, and the polysaccharide from the ciliate *Tetrahymena pyriformis* (Manners and Ryley, *Biochem. J.*, 1952, **52**, 480) as a glycogen. Molecular weight must be of minor importance. For example, the amylopectin from rubber-seed starch (D.P. 6000) binds about ten times more iodine than rabbit-liver glycogen (D.P. 30,000). (For values of D.P., see Greenwood and Robertson, *loc. cit.*)

It was possible to test the hypothesis that fine structure governed iodine-uptake when samples of " abnormal " branched α-1 : 4-glucosans became available. Samples of the water-soluble polysaccharides from sweet corn (*Zea mays*) were kindly placed at our disposal by Drs. R. L. Whistler and W. J. Whelan. The exact structural nature of these polysaccharides has been in dispute (cf. Morris and Morris, *J. Biol. Chem.*, 1939, **130**, 535; Hassid and McCready, *J. Amer. Chem Soc.*, 1941, **63**, 1132; Sumner and Summers, *Arch. Biochem.*, 1944, **4**, 7; Cameron, *Genetics*, 1947, **32**, 459; Dvonch and Whistler, *J. Biol. Chem., loc. cit.*). Whilst these materials have an average length of unit chain of 12—13 and 10—11 glucose residues respectively (*idem, loc. cit.*; Whelan, personal communication), the iodine-uptake was three to four times greater than that for a glycogen of corresponding average chain length (see Table 3), although the molecular weights were of the same order (Greenwood, unpublished work). It is, therefore, suggested that these polysaccharides have a degree of multiple branching *intermediate* between those of glycogen and amylo-

pectin, and are therefore neither in the one class nor the other. [It is of interest that Wolff, Watson, and Rist (*J. Amer. Chem. Soc.*, 1953, **75**, 4897) reached a similar conclusion from a study of the tricarbanilates of polyglucosans with different linkages.]

An abnormal rabbit-liver glycogen [shown by Haworth, Hirst, and Isherwood (*loc. cit.*) from methylation studies to have an average unit chain of 18 glucose residues] bound about five times more iodine than a normal glycogen and appeared to behave more as an amylopectin-type structure. Without additional information, it is not possible to say whether this is due to the increased average length of the external chains (*i.e.*, 12 residues) or to a variation in the degree of branching. [The abnormal character of this glycogen has been confirmed by Professor F. Smith (personal communication), who found it to possess an abnormal precipitin reaction with concanavalin-A.]

The amylopectin from wrinkled-pea starch (*var.* Perfection) has been shown to possess abnormal iodine-binding power, and an average unit chain of 36 glucose residues (Potter, Silveira, McCready, and Owens, *loc. cit.*). These authors deduced from spot tests that no amylose was present. A sample of this material, kindly provided by Dr. R. M. McCready, gave an abnormal titration curve indicating the presence of some linear material. The iodine-binding power was about six times greater than that for a normal amylopectin (Table 3).

Although data regarding the average external chain length of the amylopectins studied are not complete, the results quoted in Table 3 suggest that small variations may exist in the degree of multiple branching of this group of polysaccharides. Additional evidence might be obtained from a study of the appropriate limit dextrins (cf. Foster and Smith, *Iowa State Coll. J. Sci.*, 1953, **27**, 467), and experiments on these lines are in progress. Similar effects have been found from a study of a large number of glycogen samples (Greenwood and Manners, unpublished work).

Hence, potentiometric determinations of the amount of iodine bound by branched α-1 : 4-glucosans, in conjunction with estimations of chain length, should enable an estimate of the degree of multiple branching to be obtained if the method can be confirmed by enzymic degradative experiments (Peat and his co-workers, *loc. cit.*; Hirst and Manners, *Chem. and Ind.*, 1954, 224). Such experiments are now in progress in these laboratories.

It is of interest that, for the samples studied, the slope of the linear portion of the titration curve for a whole starch is greater than that for the corresponding isolated amylopectin. This implies that sub-fractionation has occurred, and emphasizes the importance of study of all supernatant and precipitated materials obtained during fractionation (cf. Greenwood and Robertson, *loc. cit.*).

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9.

AN INVESTIGATION OF THE POLYSACCHARIDE CONTENT OF OATS, *AVENA SATIVA* L.

By D. M. W. ANDERSON and C. T. GREENWOOD

A graded extraction procedure has been applied to the polysaccharides present in the kernels of oats, *Avena sativa* L. Analyses of the sugar, protein and ash content of the fractions obtained are reported. A method for the purification of the starch present in the kernels is given. Oxidation of the unfractionated starch with potassium metaperiodate showed that the ratio of terminal to non-terminal glucose units was 1 to 27·4. Differential potentiometric iodine titrations indicated the presence of 26·0% of amylose in the starch, and hence the average length of unit-chain in the amylopectin component was calculated to be 20·3 glucose units. Examination of the impure intermediate starch products showed that the protein impurity interfered considerably with the interaction of the starch with potassium metaperiodate.

Introduction

The polysaccharide content of many types of seed material has recently been examined Because of their importance, the common cereals, wheat, barley and maize, have attracted considerable study, and work carried out on waxy maize starch has been reviewed recently.[1] Investigations into the isolation of wheat starch from the contaminating gluten have been carried out by McMasters & Hilbert[2, 3] and by Glendenning & Wright,[4] and the cold-water extract from this cereal has been examined by several workers.[5, 6, 7, 8] McWilliam & Percival[9] have carried out a full chemical investigation of the starch from barley, and the corresponding cold-water extract has been partially separated into its components.[10, 11, 12] The cold-water extract from each of the common cereals has been shown to give several sugars on hydrolysis,[12] but the structural significance of these is not yet completely established. In the case of the

cereal, oat, little work on the polysaccharide content has been reported, although Morris[13] has claimed to have isolated lichenin and araban from the cold-water extract.

In the present work, the distribution of all the component polysaccharides in the oat kernel has been investigated, whilst special attention has been devoted to determine whether *pure* starch could be isolated in good yield. Chemical methods were avoided in the early stages of extraction to reduce possible damage to the starch granules. After removal of fats and deactivation of enzymes, oat kernels (*var.* Sun II, 1952 crop) were successively extracted with cold and hot water followed by cold and hot alkali. The fractions obtained were hydrolysed with acid, then examined qualitatively and quantitatively for the liberated component sugars ; in addition, the amount of ash, protein, and unhydrolysable material in each was determined. The starch fraction was purified free from protein, and the percentage of amylose and the ratio of non-terminal to terminal groups determined. The presence of contaminating protein in the starch was found to interfere with both these determinations. In order to study these effects in more detail, a second sample of impure starch from the same variety of oats (1953 crop) was extracted with M-sodium chloride and toluene to provide a wider range of protein-contaminated samples.

Experimental

Before analyses, samples were dried by heating *in vacuo* at 80° for several hours. Solutions were concentrated under reduced pressure at 40°. Percentages of nitrogen were found by duplicate semi-microKjeldahl determinations.

Extraction of fats from the kernels

Oat kernels (*var.* Sun II, 1952 crop) were ground to a coarse flour (Found : moisture, 11·8 ; ash, 2·22 ; protein, 14·0 ; ether-extractable substances, 7·2%), and this was exhaustively extracted with a boiling benzene–methanol mixture (2 : 1, v/v ; 1·5 l./200 g. flour).

Cold-water extraction

Defatted kernels (100 g.) were shaken vigorously with distilled water (1 l.) (five treatments, each of about 12 h.). After each extraction, the turbid extract obtained on filtration was centrifuged to give a proteinaceous starch deposit (fraction F1), and a supernatant liquid containing water-soluble material. Supernatant liquids were combined, reduced in volume and freeze-dried to yield fraction (F2).

Purification of fraction (F1) (Starch I)

This portion was treated by fractional centrifuging using the procedure detailed below for Starch II, to give fractions F1*a*, *b* and *c*.

Hot-water extraction

The residue from the cold-water extraction was stirred for 3 h. with 1 l. of water at 90°. The extract, which appeared to contain starch, was treated with thymol (2 g.) to give, after keeping at 25° for three days, a characteristic amylose–thymol complex (F3*a*). The supernatant liquid from the thymol separation yielded fraction F3*b* after freeze-drying. The residue from the first cold-water extraction was extracted four more times with hot water as before and the extracts combined, giving after freeze-drying, fraction F3*c*.

Alkaline extractions

The residue from the cold- and hot-water extractions was stirred five times with sodium hydroxide (5% w/v ; 500 ml. ; about 2 h. each time) at room temperature under nitrogen. The supernatant liquids obtained on centrifugation were brought to pH 7 with glacial acetic acid, and the resultant precipitate collected (fraction F4). Material in the solution was precipitated (fraction F6) by the addition of ethanol (2 vol.).

A similar procedure using sodium hydroxide (5%, w/v ; 500 ml. ; about 2 h. each time) at 90° in a nitrogen atmosphere gave fractions F5 and F7.

The residual material was washed free from alkali and dried (F8).

Qualitative analysis of the fractions for sugars

Samples (F1–F7) were hydrolysed with 2% sulphuric acid (2 ml./20–30 mg.) at 100° for 8 h. For (F8), the hydrolysis-time was increased to 18 h. The solutions were then neutralized with barium carbonate, the precipitated sulphate removed, and the concentrated solutions examined by partition chromatography. [Whatman No. 1 filter paper; descending method; solvent system: butanol–benzene–pyridine–water (5 : 1 : 3 : 3—top layer).] After development for 48 h., good separation of all sugars was achieved, except for fraction (F2), where the fructose and arabinose were not resolvable. Two chromatograms were examined for each sample, aldoses being located with aniline oxalate spray, and ketoses with urea oxalate. All sugars detected were identified by comparisons with control solutions.

In addition, the unhydrolysed cold-water extract was examined, when maltose, galactose, mannose, arabinose and xylose were shown to be absent.

No ribose was liberated from any fraction on hydrolysis.

Quantitative analysis of fractions for sugars

For quantitative analyses, a known weight of ribose was added as a reference sugar. After hydrolysis (as above) any non-hydrolysable material was removed, and weighed. The sugars were then separated on Whatman 3MM paper using the above solvent system at 21° for 48 h., and eluted with water from their located sites.[14] Estimations of sugar concentration were made on aliquots using Somogyi's reagent.[15] Fructose and fructosans in F2 were estimated using Arni & Percival's colorimetric method.[16]

Purification of oat starch II

Crude oat starch (Starch II, separated from the 1953 sample of oats) was purified by dispersing in 1M-sodium chloride so as to give a suspension of specific gravity 1·07, to which was added toluene ($\frac{1}{10}$ vol.). The mixture was shaken vigorously for at least 1 h., then centrifuged for 5 min. at 750 r.p.m. before the speed was slowly increased to 1200 r.p.m. over a further period of 5 min. (M.S.E. 'Major' centrifuge). The brown precipitate at the toluene–water interface was removed and discarded. The deposited material consisted of two well-defined layers; the upper (proteinaceous) layer was loosely packed and easily removed with a spatula followed by gentle washing with a jet of water. The lower layer was much whiter and very densely packed. These two layers were then separately re-suspended in 1M-sodium chloride, and the extraction procedure repeated six times. The nitrogen content of the final starch (IIa) was only 0·03%, and other fractions of higher nitrogen content were isolated at intermediate stages of the purification process.

After washing free from salt, these products were refluxed with 85% methanol (three treatments, each of 2 h.) to ensure complete removal of fatty materials, finally yielding fractions of which Starches II*a–e* are representative (for protein content see Table II).

When an aqueous suspension of Starches II*b* and *c* was passed through a column (10 × 1·5 cm.) of Zeokarb-215 resin, the nitrogen content decreased from 0·07 and 0·47% to 0·05 and 0·34% respectively (75% recovery of material). No decrease, however, was found in the case of Starch II*a*, and further treatments of II*b* and *c* with resin, and also with hot 80% ethanol, did not further reduce these percentages of nitrogen.

*Analysis of Starch II*a

The white powder consisted of birefringent granules, forming in hot water a clear paste which stained blue with iodine. Hydrolysis with 2% sulphuric acid (2 ml./20 mg.) at 100° for 7 h., yielded 99·1% of the theoretical amount of glucose (alkaline hypoiodite determinations[17] at pH 11·4[18]). There was no residue after hydrolysis, and no other sugar could be detected by chromatography. The starch had $[\alpha]_D^{18}$ + 163° (*c*, 0·5% in N-NaOH), $[\alpha]_D^{18}$ + 194° (*c*, 0·34% in 30% $HClO_4$) (Found: sulphated ash, 0·024; N, 0·03%).

Determination of the percentage of amylose

The differential potentiometric titration technique of Gilbert & Marriott[19] was employed to measure iodine-binding power. The electrometer used and the titration conditions have been previously described.[20]

Determination of the ratio of terminal to non-terminal groups [i.e. $(\overline{R})$] in the starch

The amount of formic acid liberated and the amount of periodate consumed on oxidation of the starch by potassium metaperiodate were determined as described by Anderson, Greenwood & Hirst.[21]

Results

The chromatographic analyses of the unhydrolysed cold-water extract (F2) indicated that oligosaccharides (fructosans), raffinose, sucrose, glucose and fructose (approximately 10, 15, 65, 5 and 5% of the total, respectively) were the only free sugars present. Table I shows the results of analyses of the hydrolysed fractions. The potentiometric iodine titration curve for Starch II*a* (Fig. 1) shows that the starch bound 5·0% of its own weight of iodine. If the maximum iodine binding-power of pure amylose under these experimental conditions is 19·2%,[22] then the starch contains 26·0% of amylose.

Table I

Analyses of the fractions isolated by the graded extraction procedure

Fraction	% of original material (approx.)	% Protein (N × 6·25)	% Ash	% residue after hydrolysis	Reaction with iodine	Percentages of sugars obtained on hydrolysis*: Pentosan† Oligosaccharides	Galactose	Glucose	Fructose	Arabinose	Xylose
F1*a*	45	0·3	0·03	—	+	—	—	98	—	—	—
F1*b*	9	7·9	0·12	—	+	—	—	90	—	—	—
F1*c*	14	28·4	0·31	—	+	—	—	68	—	—	—
F2	7	20·9	1·08	1·23	—	2‡	6	79	5	4	4
F3*a*	3	5·1	2·13	—	+	—	3	89	—	4	4
F3*b*	7	6·9	0·90	1·14	+	2	1	88	—	5	4
F3*c*	6	11·5	1·37	1·58	+	1	2	87	—	5	5
F4	1	68·8	4·28	18·3	—	—	—	28	—	34	38
F5	Negligible	—	—	—	—	—	—	—	—	—	—
F6	3	9·8	6·12	15·3	—	3	3	26	—	32	36
F7	2	1·4	39·4	77·3	—	—	15	30	—	25	30
F8	3	1·6	7·65	71·6	—	—	4	21	—	39	36

* Expressed as percentages of hydrolysable carbohydrate
† Incompletely hydrolysed polysaccharides
‡ This fraction also contained some hexosans

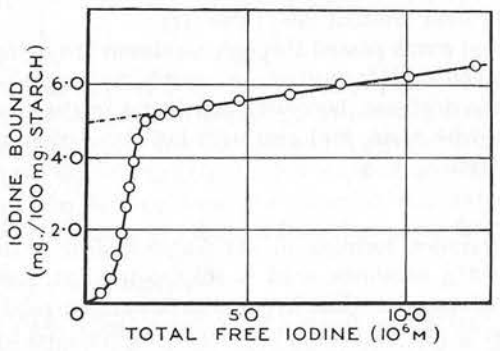

FIG. 1.—*The potentiometric iodine titration curve for Starch II*a
([I′] = 0·01M; pH = 5·85; temp. = 20°)

The results of periodate uptake showed that at 15–16° oxidation was complete in 240–318 h. The amount of formic acid liberated in this time corresponded to a value for $(\overline{R})$ of 27·4 glucose units. The average length of unit chain in the amylopectin component of the starch was hence calculated to be 20·3 glucose units. The effect of protein on the observed value of $(\overline{R})$ is shown in Table II for centrifuged and non-centrifuged samples.

Table II

Value of ($\overline{R}$) found for proteinaceous starches by periodate exidation*

Sample	% Protein	Oxidation-time at 15–16°							
		10 days		11 days		12 days		13 days	
		C	NC	C	NC	C	NC	C	NC
II*b*	0·45	29·9	29·1	29·0	28·3	28·2	27·5	27·9	27·3
II*c*	2·94	31·1	29·7	30·2	28·8	28·4	27·6	28·1	27·1
II*d*	22·7	48·8	32·8	46·5	28·0	40·5	27·8	38·6	25·3
II*e*	45·6	79·6	34·1	66·8	28·4	64·7	26·2	51·8	23·5

* Sample weight corrected for percentage protein present
C = Value obtained by titration of reaction mixture centrifuged free of oxidized granules
NC = Value obtained by titration of non-centrifuged reaction mixture

Discussion

The samples of groats studied had been *completely* freed from contaminating hulls, although a significant proportion of the original oats were green-tipped. Results similar to those of Macleod & Preece[23] have been obtained for the analysis of the free sugars in the cold-water extract of the defatted kernels. All the polysaccharide fractions obtained from the extraction scheme were contaminated with protein ; no effort was made to remove this except in the case of the starch product, where a physical method of separation was successful. The high ash content of some fractions was due to the fact that no dialysis was carried out (to avoid loss of carbohydrate) before isolation of the fractions by freeze-drying. The whole of the fructosan content of the kernels was found in the cold-water extract F2, whilst galactose, glucose, arabinose and xylose were present in the hydrolysis product of nearly all the fractions.

Complete separation of the polysaccharide material into products of varying solubility is thus difficult. As a result, the differentiation between the water-soluble and hemicellulosic materials is not sharp, but water-soluble pentosans and glucosans other than starch are present. A large percentage of the glucose in the hot-water extracts was probably present as starch (which cannot be completely separated using cold water), whilst that in the alkaline-soluble fractions was probably hemicellulosic in nature. The large amount of unhydrolysed material in F7 is accounted for by the high ash content of this fraction, whilst that for F8 is probably due to the presence of cellulosic material.

On hydrolysis, the cold-water extract F2 was shown to give rise to sugars in quantities very similar to those described by Preece & Mackenzie,[12] bearing in mind that these authors combined galactose with glucose and fructose with arabinose.

About 75% of the starch present in the kernel could be purified to a protein content of 0·3% under the mild conditions necessary to minimize degradation. A prolonged purification procedure produced a quantity of starch having a protein content of 0·19%. This percentage could not be further reduced and it would be difficult to determine whether this residual protein is chemically bound to the starch. The percentage of amylose is higher than for barley starch, but is of the same order as for wheat.[21] Further work is in progress on the isolation of the amylose from the starch fraction. The average length of unit-chain of the amylopectin component appears to be similar to that of many other starches.[21]

Examination of the samples of protein-contaminated starches showed that, with these materials, abnormal results were obtained from both periodate oxidation and potentiometric iodine titration experiments. The effect of this impurity on periodate oxidation is shown in Table II ; the value of ($\overline{R}$) obtained for protein-contaminated starches most closely approximates to the correct value only when estimations are carried out by titrating *uncentrifuged* samples of the reaction mixture to pH 6·25.[21] This technique is suitable for samples containing up to 23% protein : above this value consumption of periodate exceeds the theoretical and no reliable figure for ($\overline{R}$) can be obtained. On the other hand, the presence of even 3% of protein seriously interferes with the observed iodine-uptake. This effect will be described in detail elsewhere. These data show the necessity for *rigorous* removal of protein from polysaccharide samples before structural examinations are attempted.

For Starch I*a*, the values obtained for $(\overline{R})$ and the percentage of amylose were identical with those for Starch II*a*. Since the oats, although of the same variety, were grown in different years, possible variations in growth conditions produced no change in these characteristics of the isolated starches (compare references 21 and 24).

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THE INTERACTION BETWEEN COMPLEX ACIDIC POLYSACCHARIDES IN SOLUTION AND METAL IONS

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Several examples have been reported in the literature of the alteration of the properties of dilute solutions of neutral high polymers by the presence of metal ions. Such effects are most conveniently studied by ultracentrifugal or viscometric techniques. In the case of derivatives of cellulose in non-aqueous solvents (e.g. the acetate in methyl ethyl ketone,[1] the ethylate in benzene and ethyl acetate,[2] and the nitrate in acetone solution[3]), traces of metal ions have been found to cause aggregation. This is thought to be owing to a " cross-linking " action of the metallic ions between a few anomalous free carboxyl groups or free hydroxyl groups, either by binding or adsorption, respectively.[4]

Similar aggregative effects do not appear to have been reported for aqueous solutions of polyelectrolytes where large numbers of free charged groups are available for cross-linking. The authors have examined this effect in the case of the plant gum, *Khaya grandifolia*, which is the exudate of West African mahogany. The main structural features of the gum have been described already,[5] and it is characterized by possessing a high uronic acid content (47%).

The gum was carefully purified free from inorganic materials by several reprecipitations with ethanol from aqueous solution. In 0·15 M sodium chloride solution, the free acid behaved as a polymolecular† system when examined *immediately* in the Spinco electrically-driven ultracentrifuge. When re-examined after standing at room temperature for several days, a tendency for the appearance of polydispersity was apparent. Further experiments were carried out using a " crude " gum containing ash (10%). Ultracentrifugal examination of this sample in buffers of varying pH (4·1–9·5) and ionic strength (0·15–0·34) indicated a polydisperse system with two or three heavier components. At room temperature, the relative amounts varied with the buffer system and the lengths of time the gum had been in contact with the particular solvent. Analysis of the contaminating inorganic material showed the presence of Ca^{++}, Mg^{++}, Na^{+} and K^{+} ions. The addition of Ca^{++} (0·05M) to a solution of the ash-free gum in 0·15M sodium chloride showed an immediate formation of rapidly-sedimenting gel-material and larger aggregates on the sedimentation diagram. Other heavy metal ions appear to behave similarly. It is thought that cross-linking between carboxyl groups must occur with the formation of higher molecular weight aggregates.

If the presence of trace amounts of metal ions causes aggregation (as shown by the time-effect on sodium chloride solutions of the purified gum), it is obvious that such effects must be shown to be absent before carrying out determinations of the molecular weight of similar acidic polysaccharides, otherwise the molecular weight of aggregates will be measured.

It is of interest that Säverborn[6] found, from ultracentrifugal measurements on apple pectin in various electrolyte media, that the sedimentation constant was dependent on the electrolyte used. He did not report, however, any polydispersity effects.

Physico-chemical examinations of this type of natural charged polymer are being continued. It is hoped that detailed results of the sedimentation measurements outlined above, and other investigations carried out on this gum, will be given elsewhere.

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† The term " polymolecular " is used to describe a homogeneous polymer having a variation in molecular weight, while " polydisperse " denotes a polymer system containing more than one component

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THE MOLECULAR WEIGHT OF LAMINARIN

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Laminarin, a polysaccharide found in certain common brown seaweeds, has been isolated in two forms; as " soluble laminarin " from *Laminaria claustoni*, and as " insoluble laminarin" from *L. digitata*. Early methylation studies[1] showed the two forms to be identical and the unit chain to consist of about 20 glucose units joined by β-1 : 3-linkages. This value was confirmed by molecular weight measurements using a modified Barger's technique, and thus a short linear glucosan was suggested.[1] Recently, Cook and his coworkers[2] have carried out ultracentrifugal and diffusion measurements on the two forms, and have found a similar average degree of polymerization (28 and 33 for the soluble and insoluble laminarins respectively).

However, the concept of a simple molecule has been questioned. Mannitol[3] and a non-reducing trisaccharide containing mannitol[4] have been isolated from laminarin, while anomalous 1 : 6-linkages have been suggested from studies of alkaline degradation[5] and periodate over-oxidation.[6] In view of this work, further methylation studies have been carried out at Edinburgh.[7]

Through the courtesy of Prof. E. L. Hirst, F.R.S., and Dr. A. G. Ross, the authors have been able to examine some of their methylated samples. Measurements of molecular weight were made in benzene solution by the isothermal distillation method.[8] The results obtained are shown below:

Laminarin sample	M_n	*D.P.*	*E.G.A*
Methylated, whole	1900	9	—
„ fractionated ..	12,000	58	20
Methylated, lime-treated, whole ..	2500	12	—
„ „ fractionated	13,600	65	20

M_n = number average molecular weight; *D.P.* = average degree of polymerization; *E.G.A.* = chemical end-group assay

Thefra ctionated products had been obtained by fractional precipitation of the methylated product from chloroform solution with petroleum ether, in about 80% yield. The results show that the fractionation results in the elimination of a considerable portion of low molecular weight material, and that the fractionated products possess a *D.P.* much in excess of the end-group assay values. This is of considerable importance because it is the first clear evidence that laminarin is not a simple straight chain glucosan of about 20 units in length.

It is of interest that similar results were obtained when aqueous solutions of both the unmethylated laminarin and lime-treated laminarin were examined in the Spinco ultracentrifuge. The solutions were stable for the time of measurement required using the synthetic boundary cell technique.[9] The Schlieren patterns obtained suggested that the laminarin had a very wide range of molecular weight—including higher molecular weight material—the main bulk having a sedimentation constant of $0{\cdot}5 \times 10^{-13}$ c.g.s. units. Lime-treated laminarin, on the other hand, was more homogeneous and possessed a larger sedimentation constant ($1{\cdot}0 \times 10^{-13}$ c.g.s. units).

Without further chemical evidence it is difficult to draw any conclusions regarding the structure of the laminarin molecule, but it is certainly not simple.

Measurements on these samples are being continued, and it is hoped to publish full details of the experimental methods and results elsewhere.

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ASPECTS OF THE PHYSICAL CHEMISTRY OF STARCH

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Addendum

THE SIZE AND SHAPE OF SOME POLYSACCHARIDE MOLECULES

I. Introduction

The structure and properties of starch have formed the subject of very extensive investigations. As a result, the main structural features and the highly polymeric nature of the molecule are now well established, and starch is known to consist of chains of D-glucopyranose units joined in the main by 4 → 1-α-D-glycosidic linkages.

Many of the remaining problems, particularly those connected with fine structure and shape and size, cannot be completely solved by purely chemical methods of investigation, and these have to be supplemented by the use of physical methods. Furthermore, in some instances, the results from such physical measurements have to be correlated with those obtained from studying the action of highly purified enzymes on the starch.

This survey will cover the results of physical measurements of this type carried out to about the end of 1955.

II. Problems Inherent in Starch Chemistry

Perhaps the most important advance in the chemistry of starch was the realization of the apparent inhomogeneity of the material, followed by its fractionation into simpler components—first carried out quantitatively by Schoch[1] in 1941. Starches can, in general, be separated into at least two chemically distinguishable entities: *amylose*, a mixture of essentially unbranched chains, and *amylopectin*, a mixture of highly branched chains.[2]

(1) For a review, see T. J. Schoch, *Advances in Carbohydrate Chem.*, **1**, 247 (1945).
(2) This terminology for the two major components appears to be in general

Although there is overwhelming evidence in favor of the heterogeneous nature of starch, very real difficulties exist regarding the exact nature of the association of the two components in the granule. Consequently, it has been suggested that starch is a giant homogeneous molecule, and that the apparent separation into components and its other physical properties are simply the result of a hydrolytic or degradative action. Such theories have to be considered seriously as the concept is not unreasonable, especially in view of the fact that, in its undegraded form in the native state, wood cellulose might appear to possess an indefinitely high molecular weight.[3]

Pacsu[4] has suggested a structure for starch involving a small number of non-cyclic hemiacetal linkages, the number being presumably sufficient to account for the number of endgroups determined by the methylation method. Halsall, Hirst and Jones[5] have commented on this structure, however, and have shown it to be incompatible with the results of periodate-oxidation studies. In addition, these authors pointed out that it would be difficult to explain enzymic hydrolysis and dextrin formation on the basis of such a structure.

From the results of elementary analyses for carbon, and conversion to D-glucose, Dumazert[6] has suggested that the fundamental unit in starch is not anhydro-D-glucopyranose but $C_{12}H_{22}O_{11}$, and has postulated a structure involving 1,5-acetal cross-linkages between open chains of 1,4-linked D-glucose units. It was suggested that mild, acid hydrolysis will break these 1,5-linkages and result in amylose, amylopectin, dextrins, maltotriose, and maltose as artefacts, depending on the extent of hydrolysis. Such a structure would, however, be extremely difficult to reconcile with the result of Freudenberg's[7] kinetic measurements of the hydrolysis of starch. It would yield also an exceptionally large amount of formic acid on periodate oxidation, in view of the increased number of 1,2,3-triol groups. The experimental evidence for such a structure appears to be ex-

usage, and is recommended in the "Report on Nomenclature in the Field of Macromolecules" by the International Union of Pure and Applied Chemistry, reported in *J. Polymer Sci.*, **8**, 257 (1952). It should be noted, however, that T. J. Schoch (in "Starch and its Derivatives," J. A. Radley, ed., Chapman and Hall, London, 1953, Vol. 1, p. 123) uses *A-fraction* and *B-fraction* for *amylose* and *amylopectin*, respectively.

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(4) E. Pacsu and L. A. Hiller, *Textile Research J.*, **16**, 243 (1946); E. Pacsu, *J. Polymer Sci.*, **2**, 565 (1947).

(5) T. G. Halsall, E. L. Hirst and J. K. N. Jones, *Nature* **159**, 97 (1947). See also, E. Husemann and U. Consbruch, *Makromol. Chem.*, **5**, 179 (1950).

(6) C. Dumazert, *Bull. soc. chim. biol.*, **32**, 983 (1950).

(7) K. Freudenberg, *J. Soc. Chem. Ind.* (London), **50**, 287T (1931); K. Freudenberg and W. Kuhn, *Ber.*, **65**, 484 (1932).

tremely meager. In addition, analyses of the trimethyl ether of starch have shown conclusively[8] that the basic unit is $(C_6H_{10}O_5)_n$.

A phosphate cross-link between the components has been suggested by Sutra.[9] Many starches contain a small amount of phosphorus (less than 0.2%, based on P_2O_5) which appears to be bound, either to the amylose or the amylopectin components. Sutra postulated that an ester cross-link might occur between a phosphate group attached to the reducing end of one chain of D-glucose units and either position 2, 3, or 6 of an adjacent chain, to form an alkali-stable but acid-labile linkage. Such a linkage is not unreasonable in view of the enzymic synthesis of starch from α-D-glucopyranosyl phosphate. This theory has been elaborated by Bauer and Pacsu.[10] It does not, however, explain the cases of starches containing no phosphorus, nor the isolation from phosphorus-containing starches of D-glucose 6-phosphate.[11]

A very different structure for the starch molecule has been proposed by Blom and Schwarz[12] from their measurements of the change in optical rotation of solutions of hydrolyzed starch as a function of the reducing power, hydrolysis being carried out using acid, α-amylase, and β-amylase. It was found that the change in optical rotation in the solution per mole of hydrolyzed D-glucosidic bonds (as estimated from reducing power) was not constant, but increased during the hydrolysis, to approach a final value corresponding to the hydrolysis of one maltose molecule to two D-glucose molecules. Blom and Schwarz interpreted these data as indicating that starch possesses some type of link which is more readily hydrolyzable than are 4 → 1-α- and 6 → 1-α-D-glucopyranosidic bonds, and proposed a structure involving chains of alternating D-glucopyranose and D-glucofuranose units. This interpretation, which is in conflict with all chemical evidence,[13] has been criticized by Lindberg and by Hopkins.[14] Both of these authors stressed the difference between the effects, on the rotation, of terminal and central bonds, and Hopkins also commented that such a structure would be extremely difficult to reconcile with the isolation of maltotriose from both acidic and enzymic hydrolyses.

Although the concept of a homogeneous molecule is not unreasonable,

(8) See, for example, W. N. Haworth, E. L. Hirst and J. I. Webb, *J. Chem. Soc.*, 2681 (1928).

(9) R. Sutra, *Bull. soc. chim. France*, 294M (1950).

(10) A. W. Bauer and E. Pacsu, *Textile Research J.*, **23**, 853 (1953).

(11) T. Posternak, *Helv. Chim. Acta*, **18**, 1351 (1935); *J. Biol. Chem.*, **188**, 317 (1950).

(12) J. Blom and B. Schwarz, *Acta Chem. Scand.*, **6**, 697 (1952).

(13) Compare, for example, W. N. Haworth and E. G. V. Percival, *J. Chem. Soc.*, 1342 (1931).

(14) B. Lindberg, *Acta Chem. Scand.*, **7**, 237 (1953); R. H. Hopkins, *Nature*, **171**, 429 (1953).

not one of the theories proposed to date appears to be sufficiently sound to warrant its acceptance. The experimental proof of such a postulate is indeed extremely difficult, and can probably be tackled only by kinetic methods of investigation. These are complicated, however, by difficulties in dispersing the granular structure of starches without degradation, and by the physical instability of any starch "solution."

The size and shape of a starch granule is characteristic of the source.[15] As laid down, the granules are surrounded by a thin layer of protein, and in some storage organs are held in a heavier, cellulosic, wall structure. The exact nature of synthesis and deposition of starch in the granule is not known, but the gradual expansion of the enzyme-containing, protein sac during growth may well entail the synthesis of the amylopectin component in an essentially two-dimensional arrangement.[16] Most granules appear to be built in layers which partially or completely encircle the *hilum*.[17] These apparent layers arise from discontinuities in refractive index of the deposited material,[18] and their exact nature has not been established. The birefringent properties of the granule have been attributed by Meyer[19] to the presence of spherulites (spherical aggregations of needle-shaped crystals), which possess no definite surface and consist of small crystalline regions held together by secondary valence forces. Meyer regards the amylopectin component as primarily responsible for this crystallinity and for the general granular structure, as "waxy-starch" granules still exhibit crystallinity. The concentric shells in the granule were thought by Meyer to consist of an outer, water-resistant layer which is transformed gradually into, and is less optically dense than, a well crystallized, inner layer. Both layers are regarded as containing radially arranged spherulites, the outer, resistant one consisting mainly of amylopectin (90%) with which high molecular-weight amylose exists[20] as "mixed crystals"; the less resistant, inner layer consisting of well crystallized, low molecular-weight amylose. Meyer suggested that the spherulites are formed either by the grouping of a number of branches of the highly ramified, amylopectin molecule into radially

(15) See, for example, E. T. Reichert, "The Differentiation and Specificity of Starches in relation to Genera, Species, etc.," *Carnegie Inst. Wash. Publ. No.* **173,** Parts 1 and 2, 1913. An excellent review of the early literature on the starch granule is given by C. L. Alsberg, *Plant Physiol.*, **13,** 295 (1938).

(16) In the case of glycogen, no corresponding definite boundary conditions exist during enzymic synthesis, and hence it may have a true three-dimensional structure.

(17) The hilum is the point of intersection of two or more creases, and can be located by polarized light, under which it appears as the center of a black cross.

(18) A. Frey-Wyssling, *Z. Botan.*, **33,** 362 (1938).

(19) K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta*, **23,** 890 (1940); K. H. Meyer, *Experientia*, **8,** 405 (1952).

(20) K. H. Meyer and R. Menzi, *Helv. Chim. Acta*, **36,** 702 (1953).

oriented, crystalline bundles, or by the aligning of the outside branches of several molecules to form a "fringe micelle." Whereas some branches of one amylopectin molecule may align themselves to form part of a micelle, the rest, including the branch points, may be in the amorphous region[21] in which secondary valence forces act tangentially to hold the granule together.

This structure for the granule is generally consistent with the x-ray diffraction data as yet available. However, it is difficult to envisage how amylopectin molecules possessing the three-dimensional structure suggested by Meyer (see p. 353) can align themselves to form any proportion of "crystalline" regions; molecules with an essentially two-dimensional structure (as mentioned above) could do so far more readily.

A similar structure has been given by Baker and Whelan,[22] in which the granule is regarded as being built up of alternate layers of amylose and amylopectin, the thickness of the amylose layer decreasing from the center of the granule. This hypothesis has been criticized by Badenhuizen.[22a]

These concepts have to be correlated with the inherent helical form of the starch molecule, and attempts which have been made to determine the orientation of the portions of the molecules in the crystallites will be dealt with later (in the Section on x-ray diffraction studies—see p. 376). In this connection, the work on the orientation of synthetic crystalline polymers into spherulites containing helically arranged molecules[23] may be important.

Another useful approach to this problem has been made by Whistler and coworkers,[23a] who have presented electron micrographs of the outside structure and internal structure of the granule. Studies of the effect of aqueous leaching and of preferential hydrolysis by acids[20] and enzymes[23b] on the granule are also important.

The question of whether or not the proportions of the two components vary during growth of the granule has not been decided.[1, 24]

Starches often contain minor constituents which may profoundly in-

(21) Compare the case of elastomers and cellulose.

(22) F. Baker and W. J. Whelan, *J. Sci. Food Agr.*, **2**, 444 (1951).

(22a) N. P. Badenhuizen, *Cereal Chem.*, **4**, 286 (1955).

(23) A. Keller, *J. Polymer Sci.*, **11**, 567 (1953); L. B. Morgan, *J. Appl. Chem.* (London), **4**, 160 (1954).

(23a) R. L. Whistler, J. D. Bryd and W. L. Thornberg, *Biochim. et Biophys. Acta*, **18**, 146 (1955); R. L. Whistler and E. S. Turner, *J. Polymer Sci.*, **18**, 153 (1955).

(23b) Compare, R. M. Sandstedt, *Cereal Chem.*, **32**, Suppl. (May, 1955).

(24) Compare: K. H. Meyer and P. Heinrich, *Helv. Chim. Acta*, **25**, 1038 (1942); T. G. Halsall, E. L. Hirst, J. K. N. Jones and F. W. Sansome, *Biochem. J.* (London), **43**, 70 (1948); M. J. Wolf, M. M. McMasters, J. E. Hubbard and C. E. Rist, *Cereal Chem.*, **25**, 312 (1948).

fluence physical behavior. Many contain fatty acids, and it is *essential* that these be removed, as they interfere both in fractionation and in interaction of the starch with iodine. Fortunately, this can be accomplished easily by extraction with a water-miscible, fat solvent (for example, methanol and aqueous dioxane).[25] This procedure also removes the small amount of phosphorus present in most starches, although that in potato starch occurs as a 6-phosphate. For this reason, the behavior of potato starch in solution may well be anomalous.

It appears impossible to remove entirely the contaminating protein from a starch by purely *physical* methods of purification; the residual protein (0.2%) may well be incorporated in the granule.

The labile nature of the components necessitates that, for fundamental investigations, the starch should preferably be extracted from its botanical source, in the laboratory, under the mildest possible conditions.[26] Industrial samples of unknown origin and treatment should not be used. The characterization of the starch would appear to entail (1) dissolution of the granule without degradation, (2) fractionation without degradation, (3) complete analysis of the finer details of structure of the separated components (including the possibilities of intermediate structures between the extremes of amylose and amylopectin), and (4) the estimation of the size, shape, and molecular-weight distribution of these fractions.

A comparison of the fine structure of amylopectin with that of the similar, highly branched glycogen is very important. In this respect, investigations on the "amylopectin-type" polysaccharide synthesized by certain bacteria[27] and protozoa,[28] and on the "glycogen-type" material synthesized by some plants,[29] are particularly useful. In these and other investigations of fine structure, enzymic investigations have proved of value. However, results may be complicated by several factors; the use of inhomogeneous enzyme systems and of impure or (inadvertently) chemically modified substrates must be avoided.

(25) T. J. Schoch, *J. Am. Chem. Soc.*, **64**, 2954 (1942); R. W. Kerr, *Cereal Chem.*, **20**, 299 (1943); R. L. Whistler and G. E. Hilbert, *J. Am. Chem. Soc.*, **66**, 1721 (1944).

(26) See, for example, C. T. Greenwood and J. S. M. Robertson, *J. Chem. Soc.*, 3769 (1954).

(27) S. A. Barker, E. J. Bourne and M. Stacey, *J. Chem. Soc.*, 2884 (1950); E. J. Bourne, M. Stacey and I. A. Wilkinson, *ibid.*, 2694 (1950); P. N. Hobson and H. Nasr, *ibid.*, 1855 (1951).

(28) D. J. Manners and J. F. Ryley, *Biochem. J.* (London), **52**, 480 (1952); G. Forsyth, E. L. Hirst and A. E. Oxford, *J. Chem. Soc.*, 2030 (1953); G. Forsyth and E. L. Hirst, *ibid.*, 2132 (1953).

(29) D. L. Morris and C. T. Morris, *J. Biol. Chem.*, **130**, 535 (1939); D. L. Morris, *ibid.*, **154**, 503 (1944); W. Z. Hassid and R. M. McCready, *J. Am. Chem. Soc.*, **63**, 1632 (1941); W. Dvonch and R. L. Whistler, *J. Biol. Chem.*, **181**, 889 (1949); K. H. Meyer and Maria Fuld, *Helv. Chim. Acta*, **32**, 757 (1949).

III. The Fractionation of Starch

Most starches contain 15 to 27% of amylose (see Table II, p. 351), although higher percentages are found in lily and pea starches, and the so-called waxy or glutinous starches (from sorghum, rice, millet, barley and certain kinds of corn)[30] contain very little. The two components can be separated from (1) the granule by aqueous leaching, and (2) a solution by electrophoresis or by selective adsorption or precipitation. The first *quantitative* separation, carried out by Schoch,[1] made use of the selective precipitation of amylose from solution by polar, organic molecules. Schoch[1] has reviewed the subject to the end of 1944, and only work reported since then will be discussed here.

It is now certain that the formation of a complex of molecules of a polar, organic compound with amylose provides the most satisfactory method for fractionating starch; other methods give poor separation and, often, degradation. (Leaching of the granule has been said to be ineffective for achieving quantitative separation[1]; it certainly sub-fractionates the amylose if varying extraction temperatures are used.[31]) Although pure amylose is easily obtained, removal of amylose from the amylopectin fraction appears to be difficult. It is therefore essential that conditions of precipitation should be so chosen that the first separation gives amylopectin of maximal purity; the amylose simultaneously obtained, which may contain some amylopectin, is readily separated therefrom by reprecipitation (or "recrystallization").

It is obviously important that the fractionation products should be adequately characterized. The only accurate method for ascertaining the purity of the starch components, and also the amylose/amylopectin ratio in whole starch, is to determine potentiometrically the amount of iodine bound.[32-36a] Colorimetric methods which have been suggested[37, 38] are useful for comparative measurements, but are often not absolute. The "yield" of

(30) For a review, see R. M. Hixon and B. Brimhall in "Starch and its Derivatives," J. A. Radley, ed., Chapman and Hall, London, 1953, Vol. 1, p. 252.

(31) K. H. Meyer, P. Bernfeld, R. A. Boissonnas, P. Gürtler and G. Noelting, *J. Phys. & Colloid Chem.*, **53**, 319 (1949).

(32) F. L. Bates, D. French and R. E. Rundle, *J. Am. Chem. Soc.*, **65**, 142 (1943).

(33) Sylvia Lansky, Mary Kooi and T. J. Schoch, *J. Am. Chem. Soc.*, **71**, 4066 (1949).

(34) R. S. Higginbotham and G. A. Morrison, *Shirley Inst. Mem.*, **22**, 141 (1948).

(35) G. A. Gilbert and J. V. R. Marriott, *Trans. Faraday Soc.*, **44**, 84 (1948).

(36) D. M. W. Anderson and C. T. Greenwood, *Chemistry & Industry*, 476 (1953).

(36a) D. M. W. Anderson and C. T. Greenwood, *J. Chem. Soc.*, 3016 (1955).

(37) W. Z. Hassid and R. M. McCready, *J. Am. Chem. Soc.*, **65**, 1154 (1943).

(38) E. J. Bourne, W. N. Haworth, A. Macey and S. Peat, *J. Chem. Soc.*, 924 (1948).

amylose varies with the complexing reagent and is not therefore quantitative.

Fractionation necessitates complete dissolution of the starch, avoiding hydrolytic degradation on the one hand and retrogradation on the other. It is difficult to obtain proof of complete dissolution of the granules; perhaps the absence of gel on ultracentrifuging or on filtering through a fine-grade (G4) sintered-glass filter, are the only indications available. Much depends on the structure of the granule; intact granules are insoluble in cold water,[39] and swelling takes place in three stages as the temperature is raised. Limited swelling first occurs, and then, at a definite temperature (the gelatinization temperature), a characteristic, irreversible, rapid swelling occurs, whereupon the granules lose their birefringent properties and a small amount of material diffuses out. In the final stage, at a higher temperature, there is rapid diffusion of material from the granule and rupture of the remaining granule "sacs" (the latter are now regarded as artifacts). Microscopic examinations have emphasized the tangential swelling of radially oriented molecules in the granule,[40] which Bear and Samsa[41] regard as being due to the development of low-pressure cavities and as refuting the idea of granules behaving as small, osmotic cells.[42] The gelatinization temperature, which varies for different starches, is obviously related to the degree of packing and binding in the granule. On the Meyer concept of the granule,[19] the smaller crystallites on the surface dissolve as the temperature is raised, whereas the larger ones remain intact. The intermolecular meshes are loosened but not destroyed, and water molecules are able to penetrate the lattice and dissolve out the low molecular-weight, amylose fraction. All the crystallites dissolve at a higher temperature.

Difficulties in avoiding inadvertent degradation during fractionation have been stressed by Schoch[2] and by Bottle, Gilbert, Greenwood and Saad.[43] The effect of degradation is more serious in the case of the amylose component than for amylopectin. The only conclusive test for degradation is to measure the molecular size of the fractionation products, and viscosity measurements are the most convenient for this purpose. (Changes in observed reducing power are insufficiently sensitive.) The use of autoclaving for dispersing the granules appears to be a procedure liable to lead to deg-

(39) Treatment of granules with such reagents as liquid ammonia, liquid hydrocyanic acid, formaldehyde, formic acid, and alkalis causes swelling at room temperature, but little is known of the effects of such treatments.

(40) N. P. Badenhuizen, *Trans. Faraday Soc.*, **42B**, 255 (1946).

(41) R. S. Bear and E. G. Samsa, *Ind. Eng. Chem.*, **35**, 721 (1943).

(42) K. H. Meyer and Maria Fuld, *Helv. Chim. Acta*, **25**, 391 (1942).

(43) R. T. Bottle, G. A. Gilbert, C. T. Greenwood and K. N. Saad, *Chemistry & Industry*, 541 (1953).

radation.[33, 44] Dissolution of the granules by heating under reflux in an *oxygen-free* atmosphere has been suggested[26, 43] as a method of avoiding this. Some starches, such as those from peas, cannot be dispersed in the usual manner by refluxing or even autoclaving, and, in these instances, gelatinization of the granule by pretreatment with liquid ammonia,[45] or with sodium hydroxide[44, 46] has been suggested.

Successful methods entail precipitation of the amylose from solution as an insoluble complex, which is removed by high-speed centrifuging; the amylopectin is isolated from the supernatant liquor by precipitation with alcohol or, more satisfactorily, by *freeze-drying*. (Precipitation with alcohol does not always appear to be quantitative, and the physical form of the product obtained by freeze-drying is more satisfactory for subsequent dissolution and esterification.) The amylose can then be further purified by reprecipitation with the same or, preferably, a different complexing agent.

It is often important to examine the solids which can be isolated from the supernatant liquors obtained during a fractionation, as it would appear that subfractionation of amylose can occur.[26]

The most widely used complexing agents are alcohols (butanol, *n*-propyl alcohol and *n*-pentyl alcohol[1]). Schoch[33] now recommends the use of Pentasol, a commercial mixture of pentyl alcohols, for the first precipitation, and 1-butanol for recrystallizations. For corn (maize) starch, this avoids contamination of the amylopectin with an intermediate fraction which is sufficiently linear to be precipitated with Pentasol and yet has a degree of branching which prevents complex formation with butanol.

Whistler and Hilbert[47] have suggested that any water-soluble compound possessing either donor or acceptor groups capable of hydrogen-bond formation is a suitable complexing agent, and they have reported the use of nitroparaffins, nitrobenzene, pyridine, amyl acetate, and amyl methyl ketone (2-heptanone). Preference was given to nitroparaffins, and the use of 1-nitropropane, 2-nitropropane, and nitroethane was studied in detail. Although the yields of amylose were the same as with butanol, the maximum iodine-binding power of the products was much lower (14.2 to 18.2%) and the products were probably contaminated with amylopectin.

Thymol and cyclohexanol were found to be suitable complexing agents for the amylose of potato starch by Bourne, Haworth and Peat.[48, 49] These

(44) R. S. Higginbotham and G. A. Morrison, *Shirley Inst. Mem.*, **22**, 148 (1948).

(45) J. E. Hodge, S. A. Karjala and G. E. Hilbert, *J. Am. Chem. Soc.*, **73**, 3312 (1951); G. E. Hilbert and M. M. MacMasters, *J. Biol. Chem.*, **162**, 229 (1946).

(46) A. L. Potter, V. Silveira, R. M. McCready and H. S. Owens, *J. Am. Chem. Soc.*, **75**, 1335 (1953).

(47) R. L. Whistler and G. E. Hilbert, *J. Am. Chem. Soc.*, **67**, 1161 (1945).

(48) W. N. Haworth, S. Peat and P. E. Sagrott, *Nature*, **157**, 19 (1946).

authors have also claimed that a method involving the preferential adsorption of potato amylopectin on aluminum hydroxide gives an amylose of high quality.[50] A combination of the aluminum hydroxide and thymol methods was later reported,[51] but this procedure resulted in extremely low yields of both components, and probably caused sub-fractionation. Purer amylopectin was obtained by fractional precipitation. Hopkins and Jelinek[52] have studied the relative precipitating efficiency of butanol, thymol, and thymol followed by cyclohexanol, for potato amylose. They found little difference between the products, as judged by colorimetric measurements on the iodine complex. The author has found[26] that the use of thymol for the first fractionation, followed by recrystallization of the amylose as the 1-butanol complex, appears to be a general method for obtaining both pure amylose and amylopectin.

Higginbotham and Morrison[44] have carried out a comprehensive examination of the use of 1-butanol, pyridine, and isopentyl alcohol as fractionating agents. At a suitable concentration, pyridine was found to give results similar to those with 1-butanol, and was found preferable since hot, aqueous solutions of pyridine are better solvents for starch than is hot water or hot water saturated with 1-butanol. Isopentyl alcohol, in contrast, was less specific, yielding precipitates which were difficult to purify. As methods of dispersing starch, autoclaving and passage through a homogenizer were shown to cause degradation, and dissolution was effected by prolonged heating at 90°. Amyloses binding a high percentage of iodine were obtained by recrystallization from solutions of concentration not higher than 0.2%.

The effect of chain length on the ability of degraded amylose to form complexes was studied by Whistler and associates.[53] When amylose is hydrolyzed to a degree of polymerization of 20 to 40, it no longer forms insoluble complexes with nitrobenzene, *n*-pentyl acetate and 2-heptanone, although it still does with 1-butanol and 2-nitropropane.

The fractionation of acid- and alkali-treated starches has been reported,[54] but the products are obviously degraded. Further work is, however, necessary on the use of alkaline solutions for fractionating starch. Pacsu[10] maintains that the addition of an amylose precipitant to an alkaline dispersion

(49) E. J. Bourne, G. H. Donnison, Sir Norman Haworth and S. Peat, *J. Chem. Soc.*, 1687 (1948).

(50) E. J. Bourne, G. H. Donnison, S. Peat and W. J. Whelan, *J. Chem. Soc.*, 1 (1949).

(51) P. N. Hobson, S. J. Pirt, W. J. Whelan and S. Peat, *J. Chem. Soc.*, 801 (1951).

(52) R. H. Hopkins and B. Jelinek, *Biochem. J.* (London), **43**, 28 (1948).

(53) W. Dvonch, H. J. Yearian and R. L. Whistler, *J. Am. Chem. Soc.*, **72**, 1748 (1950).

(54) A. W. Bauer and E. Pacsu, *Textile Research J.*, **23**, 860, 864 (1953); R. W. Kerr and W. J. Katzbeck, *Die Stärke*, **5**, 2 (1953).

of starch does not cause fractionation, whereas Schoch reports that fractionation occurs.[2] Baum and Gilbert[55] have suggested that, under oxygen-free conditions, the amylopectin component of starch is insoluble in alkali, and that fractionation can be achieved by this means. The fractionation was, however, incomplete.

The use of aqueous leaching to fractionate starches has been recently reinvestigated. Schoch[1] maintains that the amylose product is badly contaminated by concurrent leaching of amylopectin, and that approximately half of the total linear component retrogrades *in situ* within the swollen granule and is consequently included in the amylopectin. This is, however, contrary to some results of other workers.[20, 55, 55a] Baum and Gilbert[55] have claimed that leaching at 100°C. in an *oxygen-free* atmosphere gives high-quality amylose and amylopectin (the latter being obtained by centrifugation of the extract). Similar results have been claimed for wheat starch.[55b] Further work on this problem is obviously necessary.

Traces of contaminating amylose in the amylopectin product of a starch fractionation are easily detected and estimated by their preferential uptake of iodine,[32, 56, 57] but removal of this amylose is difficult. Tanret[58] believed that it could be removed by selective adsorption on cotton wool, and later work supported this idea.[59-61] Schoch[1] showed, however, that Tanret's method had not removed the residual amylose, and that fatty acids extracted from the cotton wool had merely suppressed the iodine-complex formation by which amylose is detected. This conclusion was supported by Higginbotham and Morrison.[44] Meyer and Gibbons have claimed that complete purification of the amylopectin can be achieved by removing the contaminating amylose as an insoluble complex with stearic acid, obtained by shaking the crude amylopectin in aqueous solution with a suspension of stearic acid.[62] Gilbert, Greenwood and Hybart[63] have critically examined this method, using amylopectin and a mixture of amylopectin and amylose. No selective adsorption of stearic acid was found, and it was shown that the apparent purification obtained by Meyer and Gibbons was actually attributable to the retention of stearic acid. In addi-

(55) H. Baum and G. A. Gilbert, *Chemistry & Industry*, 490 (1954).

(55a) See, for example, P. Karrer and E. V. Krauss, *Helv. Chem. Acta*, **12**, 1144 (1929); R. W. Kerr and G. M. Severson, *J. Am. Chem. Soc.*, **65**, 193 (1943).

(55b) H. Baum, G. A. Gilbert and H. L. Wood, *J. Chem. Soc.*, 4047 (1955).

(56) S. A. Watson and R. L. Whistler, *Anal. Chem.*, **18**, 75 (1946).

(57) S. Nussenbaum, *Anal. Chem.*, **23**, 1478 (1951).

(58) C. Tanret, *Compt. rend.*, **158**, 1353 (1914).

(59) M. E. Baldwin, *J. Am. Chem. Soc.*, **52**, 2907 (1930).

(60) M. Samec, *Ber.*, **73A**, 85 (1940).

(61) E. Pacsu and J. W. Mullen, 2nd, *J. Am. Chem. Soc.*, **63**, 1169 (1941).

(62) K. H. Meyer and G. C. Gibbons, *Helv. Chim. Acta*, **23**, 210 (1950).

(63) G. A. Gilbert, C. T. Greenwood and F. J. Hybart, *J. Chem. Soc.*, 4454 (1954)

tion, the preferential adsorption of amylose by cellulose was found to be inefficient, and not satisfactory for the removal of residual amylose in potato amylopectin.

Methods of sub-fractionating both components have been reported. Kerr[64] has sub-fractionated maize amylose by dissolving it in ethylenediamine and gradually precipitating the amylose with ether. Using Meyer's technique, Kerr has also preferentially extracted different amylose fractions of corn starch.[65] Sub-fractionation of corn (maize) amylose[31] and of potato amylose[66] has been achieved by Meyer by aqueous leaching of the granules at 70°, followed by dissolution of the residue by autoclaving, and precipitation of residual amylose as its complex with cyclohexanol. Speiser and Whittenberger[67] carried out a fractionation by partial precipitation of amylose from 0.5% potassium hydroxide solution with isopropyl alcohol. An elaborate experimental method of sub-fractionation, depending on the fact that fractional solubility of amylose in saturated, aqueous butanol is a function of temperature and molecular size, has been described by Goodison and Higginbotham.[68] A useful method of sub-fractionating maize amylose has been developed by Schoch,[33] in which the gradual addition of 1-octanol to a solution of the polysaccharide caused preferential precipitation of the longer chains. The method developed by Ulmann[69] for the chromatographic separation of starch on an alumina column has been applied by Fischer and Settele[70] to sub-fractionating the components. Elution, using an acid buffer, showed that potato amylose and maize amylose are homogeneous, whereas tapioca amylose consists of two fractions.

Tapioca and maize amylopectins have been sub-fractionated by fractional precipitation from aqueous solution with increasing amounts of methanol,[64, 71] and potato amylopectin by preferential precipitation on electrodialysis of the iodine complex.[72] When these three amylopectins were subjected to chromatography, and eluted with a neutral buffer, all were found to consist of several sub-fractions.[70]

IV. The Chemical Characterization of the Starch Components

Early investigations on whole starch established the presence of 4 → 1-α-D-linkages between the D-glucopyranose units involved. Two chemical

(64) R. W. Kerr, *J. Am. Chem. Soc.*, **67**, 2268 (1945).
(65) R. W. Kerr, *Arch. Biochem.*, **7**, 377 (1945).
(66) K. H. Meyer and P. Rathgeb, *Helv. Chim. Acta*, **31**, 1533 (1948).
(67) R. Speiser and R. T. Whittenberger, *J. Chem. Phys.*, **13**, 349 (1945).
(68) D. Goodison and R. S. Higginbotham, *Shirley Inst. Mem.*, **24**, 235 (1950).
(69) M. Ulmann, *Kolloid-Z.*, **116**, 10 (1950); **123**, 105 (1951); *Biochem. Z.*, **321**, 377 (1951); *Naturwissenschaften*, **37**, 309 (1950); *Makromol. Chem.*, **9**, 76 (1952).
(70) E. H. Fischer and W. Settele, *Helv. Chim. Acta*, **36**, 811 (1953).
(71) A. L. Potter and W. Z. Hassid, *J. Am. Chem. Soc.*, **73**, 997 (1951).
(72) K. H. Meyer and G. C. Gibbons, *Helv. Chim. Acta*, **33**, 213 (1950).

methods have proved particularly useful in elucidating structure. These are (1) the Haworth technique of methylation followed by acid hydrolysis and separation of the component methylated sugars,[73] and (2) the quantitative estimation of the reaction products after action of periodic acid,[73a] first introduced by Jackson and Hudson.[74] These methods have been applied to the starch components and, in conjunction with molecular-weight determinations, have enabled both the configuration and the degree of branching to be estimated. Early work in this direction was carried out, and indeed the main structural features were established, using incompletely separated components.

1. *Amylopectin*

Methylation studies[75] showed that about 4% of "end group" was present, which corresponded to a length of unit chain of 27 D-glucose residues. As the molecule is non-reducing and has a molecular weight corresponding to many times this value, a highly branched structure for amylopectin was suggested by Meyer. More recently, the accuracy of the analysis (of the hydrolysis products from the methylation) has been considerably increased using the elegant chromatographic technique of Hirst and associates.[76] Results have shown that the length of unit chain varies between 17 and 26 D-glucose units[26, 77-80] for different varieties of starch.

The methylation results have been supported by those from periodate oxidation,[73a] in which the amount of formic acid liberated from the 1,2,3-triol group in each non-reducing terminal D-glucose unit is estimated. (The formic acid liberated from the reducing end group and the linear amylose component is small, and can be neglected to the first approximation.) The periodate-oxidation method has several advantages over the methylation technique; it is much quicker, less tedious, and requires about one tenth the material. However, a difficulty is that polysaccharides have a tendency[73a] to be oxidized beyond the "theoretical" stage. To avoid this, Brown, Halsall, Hirst and Jones[79] have used the sparingly soluble potas-

(73) W. N. Haworth and H. Machemer, *J. Chem. Soc.*, 2270 (1932).

(73a) See J. M. Bobbitt, this volume, page 1.

(74) E. L. Jackson and C. S. Hudson, *J. Am. Chem. Soc.*, **59**, 2049 (1937).

(75) K. H. Meyer, M. Wertheim and P. Bernfeld, *Helv. Chim. Acta*, **23**, 865 (1940); **24**, 378 (1941).

(76) E. L. Hirst, L. Hough and J. K. N. Jones, *J. Chem. Soc.*, 928 (1949); L. Hough, J. K. N. Jones and W. H. Wadman, *ibid.*, 2511 (1949); S. K. Chanda, E. L. Hirst, J. K. N. Jones and E. G. V. Percival, *ibid.*, 1289 (1950).

(77) I. C. MacWilliam and E. G. V. Percival, *J. Chem. Soc.*, 2259 (1951).

(78) W. Z. Hassid and R. M. McCready, *J. Am. Chem. Soc.*, **65**, 1157 (1943).

(79) F. Brown, T. G. Halsall, E. L. Hirst and J. K. N. Jones, *J. Chem. Soc.*, 27 (1948).

(80) G. C. Gillie and F. J. Joubert, *J. Sci. Food Agr.*, **1**, 355 (1950).

sium metaperiodate at room temperature and have kept the concentration of formic acid low, whilst Potter and Hassid[81] recommend the use of the sodium salt at 2°C. The conditions necessary for avoiding over-oxidation using potassium metaperiodate, and the details for correctly estimating the liberated formic acid potentiometrically have been critically surveyed by Anderson, Greenwood and Hirst.[82]

TABLE I

Amylose Content, and Average Length of Unit Chain, of Amylopectins

Starch source	*Amylose, %*	*Average length of unit chain*		*References*
		by methylation	*by periodate oxidation*	
Apple *var.* Newtown Pippin	0.5	—	24	83
Barley *var.* Pioneer	2	26 ± 2	24	77
Colocasia tubers	—	22	22	83a
Easter lily	—	—	27	81
Hevea brasiliensis, seed	0.4–0.7	23	24	26
Maize	—	27	25–26	75, 81
waxy	0.8	18	20	79, 82
hybrid "Amylomaize"	—	—	36	83b
Oat *var.* Sun II	0.5	—	20	84
Parsnip *var.* Hollow Crown	0.5	—	20	84
Passion fruit (*Passiflora edulis*)	1.2	17	17	80
Pea, smooth, *var.* Alaska	8.5	—	26.6	47
wrinkled, *var.* Perfection	—	—	36	47
Potato	0–1	25	24	78
var. Golden Wonder	0.3–0.9	—	22	84
var. Redskin	0.3–0.9	—	22	84
Sago	—	—	22	81
Tapioca	—	—	23	81
Wheat	—	—	23	81

These authors showed that the time required to achieve quantitative uptake of periodate ion varied from starch to starch. The oxidation time for a simple saccharide cannot therefore be taken as a standard for starch.

(81) A. L. Potter and W. Z. Hassid, *J. Am. Chem. Soc.*, **70**, 3488 (1948).

(82) D. M. W. Anderson, C. T. Greenwood and E. L. Hirst, *J. Chem. Soc.*, 225 (1955).

(82a) D. M. W. Anderson and C. T. Greenwood, *J. Sci. Food Agr.*, **6**, 587 (1955).

(83) A. L. Potter, W. Z. Hassid and M. A. Joslyn, *J. Am. Chem. Soc.*, **71**, 4075 (1949).

(83a) E. S. Amin, *J. Chem. Soc.*, 2441 (1955).

(83b) I. A. Wolff, B. T. Hofreiter, P. R. Watson, W. L. Deatherage and M. M. McMasters, *J. Am. Chem. Soc.*, **77**, 1654 (1955).

(84) D. M. W. Anderson and C. T. Greenwood, unpublished results.

Investigations of the lability of the periodate-oxidized product to alkali showed that the *p*H, during titration of the liberated formic acid, had to be very carefully controlled. Quantitative titration of formic acid, in the presence of all normal oxidation products, was shown to be complete at *p*H 6.25. (Over-titration, to *p*H 8.0, reduced the value of average unit chain by 20%.) The effect of contaminating protein on the results of periodate oxidation has also been studied in detail.[82a]

Table I shows the results of periodate oxidation and methylation applied to amylopectins from various sources. If the percentage of amylose in a whole starch is known, the length of unit chain of the amylopectin component can be calculated from the results of periodate oxidation of the whole starch (see Table II). The length of unit chain appears to depend on the botanical species, but not the variety, from which the starch was isolated. In the case of tapioca and corn amylopectins, sub-fractionation of these by precipitation with methanol, followed by periodate oxidation, showed the sub-fractions had the same degree of branching as the original amylopectins.[71] The action of periodate on whole starches and amylopectins is now so well established and accurate, that it may well completely supersede the methylation technique for pure starches, in view of its many advantages.

It was assumed from the results of early investigations that the interchain linkage is 1 → 6, since approximately equal amounts of di-*O*-methyl- and tetra-*O*-methyl-D-glucose were obtained from the hydrolyzate of the methylated starch. 2,3-Di-*O*-methyl-D-glucose was later identified.[90] Uncertainty arises in this procedure, however, in view of the difficulty of achieving complete methylation, and also the fact that tri-*O*-methyl-D-glucose tends to undergo some demethylation in methanolic hydrogen chloride; as a result, a mixture of 2,3-, 2,6-, and 3,6-di-*O*-methyl-D-glucoses is usually obtained.

Definite proof that the interchain linkage is 6 → 1-α-D has been obtained by Wolfrom and coworkers,[91] who have isolated crystalline β-isomaltose [6-*O*-(β-D-glucopyranosyl)-D-glucose] octaacetate (in 1% yield) from the mild acetolysis of waxy maize starch and have presented evidence that

(85) E. L. Hirst, J. K. N. Jones and A. J. Roudier, *J. Chem. Soc.*, 1779 (1948).

(86) W. Z. Hassid and W. H. Dore, *J. Am. Chem. Soc.*, **59**, 1503 (1937).

(87) W. G. Campbell, J. L. Frahn, E. L. Hirst, D. F. Packman and E. G. V. Percival, *J. Chem. Soc.*, 3489 (1951).

(88) E. L. Hirst and G. T. Young, *J. Chem. Soc.*, 951, 1471 (1939).

(89) C. E. Ballou and E. G. V. Percival, *J. Chem. Soc.*, 1054 (1952).

(90) C. C. Barker, E. L. Hirst and G. T. Young, *Nature*, **147**, 296 (1941).

(91) M. L. Wolfrom, J. T. Tyree, T. T. Galkowski and A. N. O'Neill, *J. Am. Chem. Soc.*, **73**, 4972 (1951).

TABLE II

Amylose Content, and Average Length of Unit Chain, of Starches

Starch source	*Amylose, %*	*Average length of unit chain*			*References*
		(a) by methylation	*(b) by periodate oxidation*	*(c) calc. for amylopectin from (b)*	
Acorn	24.0	—	—	—	85
Apple *var.* Newtown Pippin	26.5	—	—	—	83
Arrowroot	20.5	—	27.3	22	82
Banana	16.8	26	26.3	22	82
Barley *var.* Pioneer	22.0	—	29.5	23	82
Canna (*Canna edulis*)	—	27	—	—	86
Easter lily	34	—	—	—	32
Elm tree, sapwood	21.5	26	26	20	87
Hevea brasiliensis, seed	20.0	—	30–31	24	26
Horse chestnut	—	28	—	—	88
Iris germanica, tubers	27.0	—	28.0	20	82
Maize	24.0	27	26.5	20	75, 82
hybrid "Amylomaize"	50	—	—	—	83b
Maple tree, sapwood	19	30	29	22	89
Oat *var.* Sun II	26.0	—	27.4	20	82
Parsnip *var.* Hollow Crown	11.5	—	23.0	20	82
Pea, smooth, *var.* Alaska	34.5	—	—	—	47
wrinkled, *var.* Perfection	66	—	—	—	47
var. Steadfast	80	—	—	—	84
Pearl manioc	15.7	—	24.1	20	82
Potato	20.0	—	28.3	23	82
var. Golden Wonder	22.0	—	28.3	22	82
var. Redskin	21.8	—	28.3	22	82
Rice	18.5	30	27.5	22	82
Sago	25.9	—	25.0	19	82
Sweet potato	17.8	28, 34	28.2	23	79, 82
Tapioca	16.7	—	26.2	22	82
Wheat	25.0	24	26.2	20	88, 82

this compound is not formed either by resynthesis or by acid reversion.[92] This work substantiates earlier claims for isolation of isomaltose from enzymic hydrolyses of starch.[93, 94]

An alternative approach has been described by Hirst and coworkers,[85] in which periodate-oxidized amylopectin is hydrolyzed and the percentage of D-glucose in the hydrolyzate estimated chromatographically. (If a cross

(92) A. Thompson, M. L. Wolfrom and E. J. Quinn, *J. Am. Chem. Soc.*, **75**, 3003 (1953).

(93) K. Myrbäck and K. Ahlborg, *Biochem. Z.*, **307**, 69 (1940).

(94) Edna M. Montgomery, F. B. Weakley and G. E. Hilbert, *J. Am. Chem. Soc.*, **69**, 2249 (1947); **71**, 1682 (1949).

linkage occurs through C2 or C3 of a D-glucose unit in any chain, this unit will not be attacked by periodate and will appear as D-glucose on hydrolysis of the polyacetal.) The amount of D-glucose found varies between 0.5 and 1.5%,[26, 77, 85, 87, 89] which indicates that, for a chain length of about 20 D-glucose units, 75–90% of the interchain linkages must be of the 6 → 1 type. Gibbons and Boissonas[95] maintain that, after prolonged oxidation, no D-glucose is detectable and that all linkages are of the 6 → 1 type. This result is in agreement with that of Anderson, Greenwood and Hirst.[82] It should be noted, however, that from an examination of the *reduced* oxidation product (that is, the polyalcohol), Smith and coworkers[96] found 0.5% of D-glucose, and they maintain that there are some linkages in amylopectin which are not of the 6 → 1 type; the possibility of incomplete oxidation cannot be excluded.

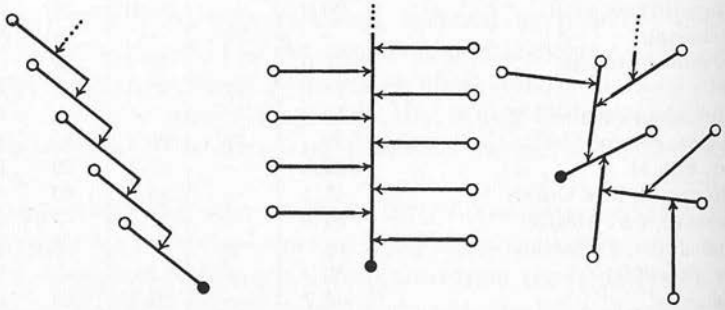

FIG. 1.—Schematic Representation of Proposed Structures for the Amylopectin Component. (Left to right: I, II, III.)

○ = terminal, nonreducing end group; ● = reducing end group; ↓ = 6 → 1-α-D linkage; ○→ = a chain of 20 to 25 4 → 1-α-D-glucose units.

In this connection, it should be noted that Wolfrom and Thompson[96a] have recently claimed the isolation of 0.1% of nigerose (a 3 → 1-α-disaccharide) from the hydrolysis of waxy-maize starch.

Various branched structures have been proposed for amylopectin. On the basis that the nonreducing end-group value shows little change with large changes in molecular weight, a laminated structure (I) (see Fig. 1) has been proposed by Haworth, Hirst and Isherwood.[97] Staudinger and Husemann,[98] after comparing the viscosities of starch solutions with those

(95) G. C. Gibbons and R. A. Boissonnas, *Helv. Chim. Acta*, **33**, 1477 (1950).

(96) M. Abdel-Akher, J. K. Hamilton, R. Montgomery and F. Smith, *J. Am. Chem. Soc.*, **74**, 4970 (1952).

(96a) M. L. Wolfrom and A. Thompson, *J. Am. Chem. Soc.*, **77**, 6403 (1955).

(97) W. N. Haworth, E. L. Hirst and F. A. Isherwood, *J. Chem. Soc.*, 577 (1937); see also T. G. Halsall, E. L. Hirst, L. Hough and J. K. N. Jones, *ibid.*, 3200 (1949).

(98) H. Staudinger and E. Husemann, *Ann.*, **527**, 195 (1937).

of cellulose derivatives having the same molecular weight, suggested structure II. Finally, Meyer and Bernfeld[99] proposed a ramified structure (III) to explain enzymic degradation.

All these structures contain different arrangements of the same basic, linear, unit chains.[100] Peat[101] has suggested that these should be termed A-, B- and C-chains, where (1) *A-chains* are side chains linked solely through their reducing group to the rest of the molecule, (2) *B-chains* are those to which A-chains are attached, although they themselves are similarly linked through their reducing group to another chain, and (3) *C-chains* carry the reducing group (that is, there can be only *one* C-chain per amylopectin molecule). The three structures differ only in the ratio of A- to B-chains. In (I), there is only one A-chain per molecule [A : B = 1:(*n*-2)], whereas in (II) there are no B-chains (A : B = ∞). Formulation III requires approximately equal numbers of A- and B-chains. Methods of differentiating between these structures will be dealt with later (see p. 384).

2. *Amylose*

Early methylation experiments indicated the presence of 0.2–0.4% of nonreducing end group in "amylose," which corresponds to a length of unit chain of about 200 to 350 D-glucose residues.[75, 78] The essentially linear nature of the molecule was first suggested by Meyer,[75] when the osmotically determined molecular weight of both maize and potato "amyloses" was found to agree with this chain length. The unbranched nature of the molecule makes chemical investigations of the structure difficult, for apart from the obvious disadvantage of estimating very small quantities of end group, degradative effects are considerable, and the amylose may be contaminated with amylopectin. For example, if a chain of 1000 units suffers only 0.5% hydrolysis of D-glucosidic bonds, the average length of unit chain is then 200 and the number-average molecular weight ($\bar{M}_n$) will be decreased similarly. If no degradation occurs, the presence of only 2% of amylopectin[102] will diminish the apparent chain length to 500, but may have little, if any, effect on $\bar{M}_n$. Methylation is therefore probably unsuitable for determining uniquely the structure of amylose.

Periodate oxidation has also been applied to this component.[79, 81, 103] Theoretically, this method should be more satisfactory than methylation, as formation of derivatives is not necessary. However, recent work suggests that the chain length of amylose comprises several thousands of D-glucose units rather than hundreds (see Section V, 2), and the accuracy of such

(99) K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta*, **23**, 875 (1940).
(100) K. Myrbäck and L. G. Sillén, *Acta Chem. Scand.*, **3**, 190 (1949).
(101) S. Peat, W. J. Whelan and Gwen J. Thomas, *J. Chem. Soc.*, 4546 (1952).
(102) Assuming the chain length = 20 D-glucose residues.
(103) A. L. Potter and W. Z. Hassid, *J. Am. Chem. Soc.*, **70**, 3774 (1948).

a chemical method in this instance is questionable. Indeed, there is not even agreement about the number of moles of formic acid liberated per mole.[104] The *availability* of the "end group" in such a long chain is extremely questionable, and over-oxidation readily occurs.

Table III shows the results of chemical analyses of amylose samples compared, where possible, with values of $\bar{M}_n$. These indicate the presence of more than one nonreducing, terminal group in some of the amylose samples. In the case of potato starch, this result is thought to be attributable to the presence of contaminating amylopectin rather than to inherent branching in the molecule.[105] Other methods of examining the fine structure of amylose, and the question of branching, will be dealt with later (see p. 381).

V. Properties of the Starch Components in Solution

1. *The Determination of Molecular Weight*

Methods which can be used to determine the size and shape of polysaccharides have been reviewed.[107] (A critical survey of these has recently been given by Sadron[108] and by Ogston.[109]) Special problems exist in the case of the undegraded starch components. In view of the branched nature of amylopectin and the large size of the amylose molecule, chemical methods of estimating size are inadequate, and it is questionable whether results are valid.[33] The free components may also aggregate in aqueous solution. Study of derivatives is therefore more convenient, and the preparation of these is an essential preliminary to estimations of molecular size.

a. Formation and Dissolution of Derivatives.—It is essential that the formation of derivatives should be accompanied by minimal degradation. Methylation is unsatisfactory from this point of view. Although procedures have been advocated[45] which cause *less* degradation, it can probably not be entirely eliminated. (For example, Greenwood and Gilbert[105] found that the osmotically determined molecular weight of a methylated amylose was one fifth that of the corresponding acetate.) It has been suggested that starch nitrates can be prepared with little degradation,[110] but no quanti-

(104) Compare: ref. 80; K. H. Meyer and P. Rathgeb, *Helv. Chim. Acta*, **31**, 1533 (1948).

(105) G. A. Gilbert and C. T. Greenwood, in course of preparation; analyses of end group carried out by E. J. Bourne, K. H. Fantes and S. Peat, *J. Chem. Soc.*, 1109 (1949).

(106) K. Hess and E. Steurer, *Ber.*, **73**, 1076 (1940).

(107) C. T. Greenwood, *Advances in Carbohydrate Chem.*, **7**, 289 (1952).

(108) C. Sadron, *Progr. Biophys. and Biophys. Chem.*, **3**, 237 (1953).

(109) A. G. Ogston, *Trans. Faraday Soc.*, **49**, 1481 (1953).

(110) G. V. Caesar and N. Goldfrank, *J. Am. Chem. Soc.*, **68**, 372 (1946).

tative evidence for this is available. Acetylation procedures,[111] using pyridine as catalyst, cause little degradation.[112] In view of this and of the high yields (>90%), acetylated products have been used almost exclusively for molecular-weight determinations.

The physical form of the component is extremely important for ease of esterification: for amylose, the order of reactivity is amylose complex > freeze-dried or freshly precipitated amylose > alcohol-precipitated, ether-dried amylose, whereas freeze-dried amylopectin is more reactive than

TABLE III

Comparison of Chemical End-group Assay and Corresponding Molecular-Weight Determinations for Amylose

Starch source	$\overline{D.P.}$[a] *from osmotic measurements*	*Unit-chain length from methylation*	*Unit-chain length from periodate oxidation*	*No. of non-reducing terminal groups/ molecule*	*References*
Barley	—	400	220	0.5	77
Easter lily, bulb	620	—	640	0.9	103
Maize	800	—	490	2.9	103
	ca. 300	*ca.* 300	—	1.0	75
Potato	200	200	—	1.0	75
	—	350	—	—	78
	930	—	980	0.8	103
Potato 1	505	250	—	2.02	105
Potato 3	258	190	—	1.36	105
Potato 5	506	101	—	5.00	105
Potato	650	247	—	2.64	106
Sago	740	—	420	3.3	103
Tapioca	1300	—	980	2.0	103
Wheat	860	—	540	2.8	103

[a] $\overline{D.P.}$ = average degree of polymerization.

alcohol-precipitated, ether-dried material. The most satisfactory method for acetylating *amylose* is that described by Higginbotham and Morrison,[44] in which the butanol–amylose complex is dehydrated with butanol and then treated with pyridine followed by acetic anhydride. Acetylation is readily achieved at room temperature, and the rate of degradation is only 0.001 bonds/initial amylose molecule/hour.[26] The method introduced by Carson and Maclay,[113] involving prior dispersion in formamide, is also successful, and has been applied by Potter and Hassid[103] to amylose which had been freshly precipitated from alkaline solution. Of other methods

(111) General methods for acetylating whole starch have been reviewed by R. L. Whistler, *Advances in Carbohydrate Chem.*, **1**, 279 (1945).

(112) K. H. Meyer, P. Bernfeld and E. Wolff, *Helv. Chim. Acta*, **23**, 854 (1940).

(113) J. F. Carson and W. D. Maclay, *J. Am. Chem. Soc.*, **68**, 1015 (1946).

available, that of Mullen and Pacsu,[114] requiring dispersion of the starch in aqueous pyridine followed by removal of water as the pyridine–water azeotrope and then addition of acetic anhydride, has been slightly modified by Cleveland and Kerr,[115] but still involves prolonged heating and may cause degradation.

The acetylation of *amylopectin* with pyridine and acetic anhydride presents more difficulty, even when using freeze-dried material,[26] and the most satisfactory method is that involving prior dispersion in formamide, after which esterification occurs readily at room temperature.

The ease with which dissolution of the acetylated products can be achieved is affected by the method of isolation. In the author's experience, drying of the acetate with alcohol and ether results in apparent "insolubility" (even though the product was soluble at one stage of the purification process), and should be avoided. Drying, under diminished pressure, of the product precipitated by petroleum ether is sufficient. Chloroform is probably the best solvent. Nitroethane, tetrachlorethane, 2,4-pentanedione, pyridine, methyl acetate, ethyl acetate, and benzene, which have also been suggested, have disadvantages in that either they are unstable or they may cause aggregation in solution.[44, 115, 116]

b. Methods of Determining Molecular Weight.—Measurements of osmotic pressure have been quite widely used for determining the molecular weight of the starch components. Several types of osmometer are available. These include the dynamic Fuoss–Mead[117] and Zimm–Myerson[118] instruments and the essentially static instrument of Gilbert, Graff-Baker and Greenwood.[119] The latter osmometer is especially suitable for rapid and accurate measurements of the very small osmotic pressures involved. No diffusion of solute occurs with undegraded starch components, and consequently the most satisfactory membranes are those which allow a rapid rate of solvent permeation. "Gel cellophane" membranes are particularly useful. Some possible sources of error in osmometry, the most important of which is the neglect of an appropriate density correction,[119] have been discussed recently.[120] The magnitude of the density correction increases with increase in molecular weight (and hence cannot be ignored, particularly in the case of amylopectin) and is important when comparisons are made between

(114) J. W. Mullen, 2nd, and E. Pacsu, *Ind. Eng. Chem.*, **34**, 1209 (1942).

(115) F. C. Cleveland and R. W. Kerr, *J. Am. Chem. Soc.*, **71**, 16 (1949).

(116) R. S. Higginbotham, *Shirley Inst. Mem.*, **24**, 221 (1950).

(117) R. M. Fuoss and D. J. Mead, *J. Phys. Chem.*, **47**, 59 (1943); C. R. Masson and H. W. Melville, *J. Polymer Sci.*, **4**, 323 (1949).

(118) B. H. Zimm and I. Myerson, *J. Am. Chem. Soc.*, **68**, 911 (1946).

(119) G. A. Gilbert, C. Graff-Baker and C. T. Greenwood, *J. Polymer Sci.*, **6**, 585 (1951).

(120) W. N. Broatch and C. T. Greenwood, *J. Polymer Sci.*, **14**, 593 (1954).

results from different osmometers.[121] Association of derivatives in solution has also to be avoided.[115, 116]

Within experimental error, a linear relationship is usually found[121a] for the variation of π/c with c for amylose acetate in chloroform solution,[26, 115, 116, 122] although Potter and Hassid[103] evaluated their results by plotting π/c against c^n (where $n = 1.39$). In the case of amylopectin acetates dissolved in chloroform, a negative slope of the π/c *versus* c curve at infinite dilution was obtained by Kerr, Cleveland and Katzbeck.[123] This behavior is not general,[26] and Potter and Hassid[103] have again used the method of plotting π/c *versus* c^n (where $n = 2.25$).

The absolute determination of the *limiting viscosity number*[124] $[\eta]$ requires corrections for (1) kinetic energy, and (2) the variation of specific viscosity with shear. Both of these corrections are extremely important.[125] Kinetic-energy corrections may be large, and are not necessarily zero for a solvent-flow time of about 100 seconds. The effect of shear, which becomes more important as $[\eta]$ increases, has not yet been investigated in the case of the starch components, although presumably it will be the more important for amylose. As these corrections have not been applied to much published work, it is extremely difficult to place the relationship between $\bar{M}_{n,w}$ and $[\eta]$ on an absolute *quantitative* basis. The data from all viscosity results quoted in the present article are expressed using concentration units of g./ml.

Viscometric manipulations are simplified considerably by using the modified Ubbelohde viscometer described by Davis and Elliott.[26, 126] This type of capillary viscometer can be modified to enable an estimate of the shear correction to be made,[127] although a Couette viscometer[128] is more satisfactory for this purpose.

(121) For example, the correction is often negligible in a Zimm–Myerson osmometer, but considerable in a Fuoss–Mead instrument.

(121a) For definitions of the mathematical symbols, see Ref. 107.

(122) J. F. Foster and R. M. Hixon, *J. Am. Chem. Soc.*, **66**, 557 (1944).

(123) R. W. Kerr, F. C. Cleveland and W. J. Katzbeck, *J. Am. Chem. Soc.*, **73**, 111 (1951).

(124) This term has been suggested (compare Ref. 2) to replace intrinsic viscosity; $[\eta] = \lim_{c \to 0} (\eta_{sp}/c)$, concentrations being expressed in g./ml. instead of g./dl.

(125) For a discussion, see: G. de Wind and J. J. Hermans, *Rec. trav. chim.*, **70**, 521, 615 (1951); W. R. Krigbaum and P. J. Flory, *J. Polymer Sci.*, **11**, 37 (1953) and previous papers quoted there; S. Newman, L. Loeb and C. M. Conrad, *ibid.*, **10**, 463 (1953); F. Bueche, *J. Chem. Phys.*, **22**, 1570 (1954); P. Goldberg and R. M. Fuoss, *J. Phys. Chem.*, **58**, 648 (1954).

(126) W. E. Davis and J. H. Elliott, *J. Colloid Sci.*, **4**, 313 (1949).

(127) J. Schurz and E. H. Immergut, *J. Polymer Sci.*, **9**, 279 (1952); T. G. Fox, J. C. Fox and P. J. Flory, *J. Am. Chem. Soc.*, **73**, 1901 (1951).

(128) A. G. Ogston and Jean E. Stanier, *Biochem. J.* (London), **53**, 4 (1953).

It should also be noted that the suggestion has been made that, at extremely low concentrations (<0.1 g./100 ml. of solution), a negative slope occurs in the η_{sp}/c *versus* c curves.[129] If this is a general phenomenon, a radical alteration in viscometric technique may be necessary.

The viscosity of polymer solutions has been considered theoretically by Flory,[130] but although this theory has been applied to cellulose esters,[131] no applications have yet been made in the case of the starch components. Theoretical predictions of the effect, on $[\eta]$, of branching in a polymer molecule have been made,[132] and this may be of importance with regard to the viscometric behavior of amylopectin.

Light-scattering measurements are of great intrinsic value for studying the starch components, but few measurements have been carried out. Recently, the question of the absolute value of the standards used in turbidimetry has been discussed,[133] and several new instruments have been described.[133, 134] It is to be hoped that more use will be made of this method in the future.

Few sedimentation or diffusion measurements have been made on the starch components, although methods of estimating polymolecularity of samples from sedimentation[135] and diffusion[136] measurements have been evaluated. The molecular-weight distribution can also be obtained from comparisons of $\bar{M}_n$, $\bar{M}_w$, and $\bar{M}_z$,[137] and more directly by fractional pre-

(129) D. J. Streeter and R. F. Boyer, *J. Polymer Sci.*, **14**, 5, 124 (1954); W. R. Moore and J. Russell, *ibid.*, **9**, 472 (1952); see also, O. E. Öhrn, *ibid.*, **17**, 137 (1955); *Acta Chem. Scand.*, **8**, 1303 (1954); D. J. Streeter, *J. Polymer Sci.*, **17**, 154 (1955).

(130) P. J. Flory and T. G. Fox, *J. Am. Chem. Soc.*, **73**, 1904, 1909, 1915 (1951).

(131) P. J. Flory and L. Mandelkern, *J. Am. Chem. Soc.*, **74**, 2577 (1952).

(132) W. Kuhn and H. Kuhn, *Helv. Chim. Acta*, **30**, 1233 (1947); B. H. Zimm and W. H. Stockmayer, *J. Chem. Phys.*, **17**, 1301 (1949); C. D. Thurmond and B. H. Zimm, *J. Polymer Sci.*, **8**, 477 (1952).

(133) H. J. L. Trap and J. J. Hermans, *Rec. trav. chim.*, **73**, 167 (1954); S. H. Maron and R. L. H. Lou, *J. Polymer Sci.*, **14**, 29, 273 (1954); Sylvanie Guinand and J. Tonnelat, *J. chim. phys.*, **51**, 539 (1954); R. W. Fessenden and R. S. Stein, *J. Chem. Phys.*, **22**, 1778 (1954).

(134) B. A. Brice, M. Halwer and R. Speiser, *J. Opt. Soc. Amer.*, **40**, 768 (1950); D. A. I. Goring and P. Johnson, *Trans. Faraday Soc.*, **48**, 367 (1952); P. Bosworth, C. R. Masson, H. W. Melville and F. W. Peaker, *J. Polymer Sci.*, **9**, 565 (1952); G. V. Schulz, H. J. Cantow and G. Meyerhoff, *ibid.*, **10**, 79 (1953); D. A. I. Goring, *Can. J. Chem.*, **31**, 1078 (1953); L. Harvey and D. Cleverdon, *J. Sci. Instr.*, **31**, 274 (1954); S. R. Caplan, *ibid.*, **31**, 295 (1954).

(135) R. L. Baldwin and J. W. Williams, *J. Am. Chem. Soc.*, **72**, 4325 (1950); J. W. Williams, R. L. Baldwin, Winifred M. Saunders and P. G. Squire, *ibid.*, **74**, 1542 (1952); L. J. Gosting, *ibid.*, **74**, 1548 (1952); R. L. Baldwin, *ibid.*, **76**, 402 (1954); J. W. Williams and Winifred M. Saunders, *J. Phys. Chem.*, **58**, 854 (1954).

(136) P. A. Charlwood, *J. Phys. Chem.*, **57**, 125 (1953); G. Herdan, *Kolloid-Z.*, **133**, 131 (1953).

(137) G. Herdan, *J. Polymer Sci.*, **10**, 1 (1953).

cipitation. In the latter method, the theoretical principles involved have been discussed.[138] Polymolecularity has been estimated from light-scattering measurements.[138a]

All these methods and theoretical advances should be applicable to the starch components, for obtaining accurate estimates of their molecular weight and its distribution.

c. Solution of Free Components.—Notwithstanding the anomalous behavior of the free components in aqueous solution, viscosity measurements in 1 *M* potassium hydroxide[33, 139] are useful criteria of relative size when careful conditions of dispersion are used and the time of examination is short. The ease with which dissolution of amylopectins is achieved may well be related to the relative molecular sizes of this component. (It has, however, been suggested that, under oxygen-free conditions, amylopectin is insoluble in potassium hydroxide solution.[55]) Other solvents for the free components include hydrazine hydrate[112] and ethylenediamine,[140] although the use of these has been criticized on the grounds that degradation may occur.[33]

2. *Amylose and its Derivatives*

a. Associative and Degradative Effects in Aqueous Solution.—The unsubstituted amylose component is unstable in aqueous solution, and tends to aggregate and be precipitated (or "retrograde") from solution.[140a] It may be possible to stabilize the amylose by formation of the characteristic iodine complex, and consequently the molecule must be able to assume several configurations in solution. The suggestion has been made that, in its stable form, this is a *helix*,[141] whereas in retrogradation, the molecular chains must align themselves to form crystallites, which can then grow to form visible aggregates. Probably in aqueous solution there is an equilibrium as follows.

"Aggregated helices" $\rightleftharpoons$ helical configuration $\rightleftharpoons$ linear configuration $\rightleftharpoons$ aggregated linear chains

(138) E. V. Sayre, *J. Polymer Sci.*, **10**, 175 (1953); R. F. Boyer, *ibid.*, **9**, 197 (1952).

(138a) H. Benoit, A. M. Holtzer and P. Doty, *J. Phys. Chem.*, **58**, 635 (1954).

(139) I. A. Wolff, Laeta J. Gundrum and C. E. Rist, *J. Am. Chem. Soc.*, **72**, 5188 (1950).

(140) J. F. Foster and R. M. Hixon, *J. Am. Chem. Soc.*, **65**, 618 (1943).

(140a) In contrast to cellulose, amylose appears to be relatively insoluble in cuprammonium. Recently, it has been suggested that this is related to the D-glucopyranoside ring *conformations* [see, R. E. Reeves, *J. Am. Chem. Soc.*, **76**, 4595 (1954)]. Variation in ring conformation may be a secondary factor in some solution phenomena of amylose.

(141) See: C. S. Hanes, *New Phytologist*, **36**, 101, 189 (1937); K. Freudenberg, E. Schaaf, G. Dumpert and F. Ploetz, *Naturwissenschaften*, **27**, 850 (1939); G. V. Caesar and M. L. Cushing, *J. Phys. & Colloid Chem.*, **45**, 776 (1941).

Intermediate forms must also exist, and, indeed, structural models of the amylose molecule exhibit considerable flexibility. Evidence for "aggregated helices" (very stable aggregates of amylose molecules) has been provided by Paschall and Foster[142] from light-scattering measurements on amylose in solvents of varying *p*H.

Retrogradation is accompanied by opalescence, increased resistance to enzymic degradation, and a decrease in viscosity. Little quantitative work on this phenomenon has been carried out. Whistler and Johnson,[143] from measurements of the weights of amyloses remaining in solution, showed that the rate of retrogradation was in the order: potato < maize < wheat. The rate of retrogradation for a series of acid-hydrolyzed amyloses passed through a maximum and it was thought that a critical size exists at which retrogradation is a maximum; molecules both smaller and larger than this will retrograde more slowly. Similar results were obtained by Schoch,[33] from turbidimetric measurements of the time taken for various subfractions of maize amylose to retrograde. The retrogradation time was inversely proportional to the chain length until a certain critical value was reached, below which the molecules were too small to crystallize. The lower retrogradation tendency of potato and tapioca starches was thus related to their larger size (molecules too large to orientate themselves completely) and not to their possessing a branched structure.

Hydrogen ions or hydroxyl ions readily catalyze the degradation of 4 → 1-α-D bonds in amylose, and any inadvertent degradation of this nature has naturally to be avoided. Since, however, chemical methods cannot account for more than 98–99 % of the linkages present in amylose, a kinetic study of this type of degradation may give information regarding the presence of any anomalous linkages.[144]

The rate of hydrolysis of amylose in 7.7 *N* hydrochloric acid has been measured by Swanson and Cori,[145] who, from comparison of this result with that for amylopectin, concluded that the 4 → 1-α-D bonds are less stable than the 6 → 1-α-D type.

The degrading effect of alkali has been known for some time. Schoch, Wilson and Hudson[146] concluded, from the fact that methyl β-maltoside is stable when heated in 0.1 *M* caustic soda for considerable periods of time, that alkaline degradation of starch occurs only through the reducing end

(142) E. F. Paschall and J. F. Foster, *J. Polymer Sci.*, **9**, 73, 85 (1952).

(143) R. L. Whistler and C. Johnson, *Cereal Chem.*, **25**, 418 (1948).

(144) Compare with cellulose: G. V. Schulz and E. Husemann, *Z. Naturforsch.*, **1**, 268 (1946).

(145) Marjorie A. Swanson and C. F. Cori, *J. Biol. Chem.*, **172**, 797 (1948). See also, M. L. Wolfrom, E. N. Lassettre and A. N. O'Neill, *J. Am. Chem. Soc.*, **73**, 595 (1951).

(146) T. J. Schoch, E. J. Wilson, Jr., and C. S. Hudson, *J. Am. Chem. Soc.*, **64**, 2871 (1942).

group. This end unit was thought to react in an enolic form before the second D-glucose was attacked. Physical measurements later showed that a random degradation of 4 → 1-α-D bonds must also occur. For example, if amylose is dissolved in 0.1 *M* alkali for any length of time at room temperature, the specific viscosity of the solution decreases. Whistler and Johnson[143] showed that this degradation was reduced to a minimum if dissolution in alkali was achieved at 0°C. in a nitrogen atmosphere. Rist[139] has suggested, however, that, at 0°C., a nitrogen atmosphere is unnecessary, as less than a 1% decrease in limiting viscosity number was found for some amyloses after 24 to 48 hr. at this temperature.

A study of the degradation occurring in both neutral and alkaline solution has been carried out by Bottle, Gilbert, Greenwood and Saad.[43] Measurements of the limiting viscosity number were used to follow any degradative effects. Potato amylose was stable in aqueous solution and in 0.2 *M* alkali at 100°C. if oxygen was excluded. In the presence of oxygen, a slow decrease in [η] occurred in aqueous solution and a very rapid decrease in alkali. Oxidative degradation was appreciable in neutral, buffered solutions, and increased with increase in buffer-ion concentration. This may point to simple nonoxidative hydrolysis. It was also suggested that an intermediate stage may occur in the degradation, as amylose which had been prepared without taking precautions to exclude oxygen contained on the average one bond per molecule which was selectively broken during acetylation with glacial acetic acid in the presence of trifluoroacetic anhydride. All such bonds were broken during the first treatment. Although these phenomena might be explicable in terms of the aggregation–disaggregation effects discussed by Foster,[142, 147] such an explanation is improbable in view of the fact that prolonged heating in alkali (in hydrogen) gave no change in [η], and, in the presence of oxygen, [η] fell unlimitedly.

Baum and Gilbert[148] have recently suggested that potato amylose contains "oxygen-sensitive" bonds. Starch and the amylose component, which had been prepared under oxygen-free conditions, showed an initial very rapid breakdown when heated in aqueous solution in the presence of oxygen (this was then followed by the slower oxidative degradation described above), although both products were stable in the absence of oxygen. The amylopectin component did not contain any of these bonds.

Amylose is thus extremely susceptible to degradation in the presence of hydroxyl ion. Two mechanisms may be involved: (1) a stepwise degradation caused by the reducing end-group's undergoing enolization followed by degradation to acidic products (this must occur in the presence or absence of oxygen), and (2) a random, catalytic, hydrolytic scission of 4→1-α-D

(147) J. F. Foster and E. F. Paschall, *J. Am. Chem. Soc.*, **75**, 1181 (1953).
(148) H. Baum and G. A. Gilbert, *Chemistry & Industry*, 489 (1954).

bonds (which does not occur in the absence of oxygen). It might be presumed, by analogy with the behavior of cellulose, that the hydrolytic scission is preceded by oxidation of a few of either the C2-, C3-, or C6-hydroxyl groups in the amylose to carbonyl groups, that is, by formation of an "oxyamylose." (Very few such groups are necessary in order to produce the observed effects.) The introduced β-alkoxyketone groups are then extremely labile to alkali.[149] The mechanism of the preliminary oxidation stage is not known, but a free radical mechanism catalyzed by, for example, trace metals or dyes may be responsible, and as such may well unavoidably occur during the isolation of starch.

The possibility of this type of experiment's indicating the presence of an anomalous unit (for example, furanose D-glucose, or D-xylose) or linkage (for example, $1 \rightarrow 2$- or $1 \rightarrow 3$-type) cannot be excluded, and it may be noted that der Wyk and Schmorak[150] have suggested that treatment with alkali preferentially breaks some anomalous cross-linkages occurring in bacterial cellulose. Further experiments are necessary, and in view of the added complications when dealing with alkaline solutions, the study of acidic hydrolyses may be more satisfactory.

To avoid inadvertent degradation of this kind during isolation and fractionation of the starch, an oxygen-free atmosphere appears essential. Perhaps the use of an oxidation inhibitor (for example, hydroquinone, quinol, or *N*-phenyl-2-naphthylamine) would also be suitable.

b. Molecular Weight and its Distribution.—The results of determinations of the molecular weight of acetylated amyloses from various sources are shown in Table IV. It can be seen that a considerable variation in $\overline{D.P.}$ has been found, even with amyloses from the same source. Generally, it appears that the values for potato, sago, and tapioca amyloses are larger than those from other sources. Although it is not improbable that the $\overline{D.P.}$ may depend on the source of the amylose, the variation in size for amyloses from the same source is most likely to be due to the use of samples which have suffered inadvertent degradation during isolation. From this point

(149) Compare: J. Kenner and G. N. Richards, *J. Chem. Soc.*, 2240 (1953); J. Kenner and W. M. Corbett, *ibid.*, 2245 (1953).

(150) A. J. A. der Wyk and J. Schmorak, *Helv. Chim. Acta*, **36**, 385 (1953).

(151) K. H. Meyer, P. Bernfeld and W. Hohenemser, *Helv. Chim. Acta*, **23**, 885 (1940).

(152) T. J. Fox, *Dissertation, Columbia Univ.*, New York (1943); quoted in Ref. 115.

(153) B. A. Dombrow and C. O. Beckmann, *J. Phys. & Colloid Chem.*, **51**, 107 (1947).

(154) W. N. Broatch and C. T. Greenwood, unpublished result. The potato amylose was prepared by R. T. Bottle and G. A. Gilbert, see Ref. 43.

(155) R. W. Kerr and F. C. Cleveland, *J. Am. Chem. Soc.*, **74**, 4036 (1951).

of view, the large value of $\overline{D.\ P.}$ for potato amylose, isolated under rigorous conditions to avoid degradation, is noteworthy.

Several investigations of the molecular weight of subfractions of amyloses

TABLE IV

The Results of Molecular-Weight Determinations on Acetylated Amyloses

Starch source	$[\eta]$ M KOH[a]	*Molecular-weight method*[b]	*Solvent*	*Molecular weight*	$\overline{D.\ P.}$	*References*
Apple *var.* Newtown Pippin	98	O. P.	$CHCl_3$	160,000	560	83
Easter lily	106	O. P.	$CHCl_3$	180,000	620	103
Hevea brasiliensis, seed	215	O. P.	$CHCl_3$	440,000	1500	26
Maize	—	O. P.	$C_2H_2Cl_4$	78,000	270	151
	123	O. P.	$CHCl_3$	230,000	800	103
	—	O. P.	$CHCl_3$	125,000–143,000	435–495	115
	—	O. P.	$C_2H_5NO_2$	197,000	685	116
	—	O. P.	$CHCl_3$	75,000	260	122
	—	S. & D.	MeOAc	156,000	540	152
	—	S. & D.	MeOAc	108,000	376	153
Pea, smooth, *var.* Alaska	176	O. P.	$CHCl_3$	220,000	770	46
wrinkled, *var.* Perfection	134	O. P.	$CHCl_3$	180,000	630	46
Potato	—	O. P.	$C_2H_5NO_2$	326,000	1130	116
	195	O. P.	$CHCl_3$	260,000	930	103
	—	S. & D.	MeOAc	69,000	240	153
	406	O. P.	$CHCl_3$	1,100,000	3800	154
	—	O. P.	$CHCl_3$	245,000	850	155
Sago	113	O. P.	$CHCl_3$	220,000	760	103
	—	O. P.	$C_2H_5NO_2$	340,000	1180	116
Tapioca	225	O. P.	$CHCl_3$	370,000	1300	103
	—	O. P.	$C_2H_5NO_2$	766,000	2660	116
	—	O. P.	$CHCl_3$	133,000	460	122
	—	S. & D.	MeOAc	187,000	649	153
	—	O. P.	$CHCl_3$	302,000	1050	155
Wheat	154	O. P.	$CHCl_3$	230,000	800	103

[a] Temperature of measurement varies between 22.5 and 35°C. [b] O. P. = osmotic-pressure measurements; S. & D. = sedimentation and diffusion measurements.

have been carried out. Schoch,[33] from studies of the limiting viscosity numbers of sub-fractions of tapioca, potato, and corn (maize) amyloses found that the frequency distribution curves indicated that the samples consisted of a homologous series of linear chains. Potter and Hassid[156] have compared the osmotically determined molecular weight of Schoch's corn (maize) and potato amylose sub-fractions with the results from per-

(156) A. L. Potter and W. Z. Hassid, *J. Am. Chem. Soc.*, **73**, 593 (1951).

iodate oxidation. They found that the number of nonreducing terminal groups per molecule was unity for the potato amyloses, but greater than unity for the maize. Although some amylopectin was present in these latter samples, as shown by refractionation experiments, the maize sub-fractions were presumed to possess a degree of branching, which appeared to increase with increase in molecular weight (1.7 branches/molecule when $\overline{\text{D. P.}}$ = 670, 7.2 branches/molecule when $\overline{\text{D. P.}}$ = 1150). The relationship $[\eta] = 6 \times 10^{-4} \times M$, for the acetates in chloroform solution at 25°C., was suggested. Speiser and Whittenberger's results[67] also indicated a wide range in molecular weight, whilst Kerr[64, 115] found that the $\overline{\text{D. P.}}$ of maize sub-fractions varied between 260 and 675.

The molecular weights of the products of an extensive fractionation of very pure maize, sago, and tapioca amyloses have been determined by Goodison and Higginbotham.[68] The sub-fractions obtained by fractional precipitation of the amylose–butanol complex were acetylated and their molecular weights determined from osmotic pressure measurements. Sago ($\overline{\text{D. P.}}$ = 1180) and tapioca ($\overline{\text{D. P.}}$ = 1300) amyloses were separated into 15 fractions, and the differential distribution curves showed sharp maxima; the $\overline{\text{D. P.}}$ of 40% of sago amylose was between 800 and 900 D-glucose units, whilst 50% of the tapioca amylose had a $\overline{\text{D. P.}}$ between 2500 and 3000 units. From the graph of $\ln[\eta]$ *versus* $\ln M$ for these acetates in nitroethane at 25°C., the following relationships were suggested.

$$[\eta] = 49 \times 10^{-2} \times M^{0.44} \text{ for sago.}$$

$$[\eta] = 3.8 \times 10^{-2} \times M^{0.65} \text{ for tapioca.}$$

$$[\eta] = 16 \times 10^{-2} \times M^{0.87} \text{ for maize.}$$

This large variation was explained in terms of branching in at least two of the amyloses.

Meyer[31] has separated maize and potato amyloses into two fractions each, by (1) aqueous leaching, followed by (2) dissolution and precipitation of the remainder. His results of colorimetric, end-group determinations of size were:

maize amylose	(1) M.W. =	40,000; $\overline{\text{D. P.}}$ =	250
	(2) M.W. =	340,000; $\overline{\text{D. P.}}$ =	2100
potato amylose	(1) M.W. =	32,000; $\overline{\text{D. P.}}$ =	200
	(2) M.W. =	110,000; $\overline{\text{D. P.}}$ =	700,

and he suggested that two distinct ranges of molecular weight are present in amylose samples. Similarly, Kerr[65] has separated maize amylose into three fractions by carrying out aqueous leaching at two temperatures before

precipitating the remainder. From measurements of the limiting viscosity numbers of the products in 1 *M* potassium hydroxide, and from the variation in slopes of the η_{sp}/c *versus* c curves, he suggested that a variation in branching (not due to contaminating amylopectin) occurred between products solubilized at low and high temperatures.[157]

The molecular weights of four sub-fractions of maize amylose, which had been "disaggregated" by treatment with alkali, have been determined by Foster and Paschall[147] from light-scattering measurements. Sub-fractionation was achieved by crystallization from 15% aqueous pyridine, and disaggregation by dissolving in 1 *M* alkali, neutralizing to *p*H 6 to 6.5, allowing to stand at room temperature until gross retrogradation had occurred, and then dissolving the retrograded material in 0.5 *M* alkali. The results shown in Table V were interpreted as showing that aggregation was a general phenomenon, and that the treatment with alkali had disaggregated

TABLE V

Effect of Alkali Treatment on Maize Amylose Sub-fractions[147]

Fraction	*Molecular Weight*	
	before treatment	*after treatment*
1	740,000	58,000
2	900,000	53,000
3	1,000,000	120,000
4	1,500,000	57,000

the amylose into a truly molecular dispersion. The possibility that alkaline degradation had occurred cannot however be ignored, particularly in view of the fact that Higginbotham[68] achieved a molecular-weight fractionation using a very similar procedure, and it is improbable that any aggregation would persist throughout acetylation.

c. Molecular Shape.—Relatively few measurements of molecular shape have been carried out. Foster and Hixon[122, 140] have found the value of α in the modified Staudinger equation to be equal to 1.5 both for amylose acetates in chloroform solution and for the unsubstituted amyloses in ethylenediamine. This high value of α was interpreted as suggesting that the amylose molecule behaves as a rigid rod in solution. However, these results are not in agreement with those of Potter and Hassid, who found $\alpha = 1$, nor with those of Goodison and Higginbotham,[68] who have found that the value of α varies between 0.44 and 0.87 for the acetates in nitroethane.

(157) R. W. Kerr, "The Chemistry and Industry of Starch," Academic Press Inc., New York, N. Y., 2nd Edition, 1950.

Peterlin[158] has shown that the viscometric data for potato amylose acetate in chloroform solution can be readily interpreted in terms of a random-coil model for the molecule, in which there is hindered rotation at the oxygen atom of the glucosidic linkage.

Dombrow and Beckmann[153] have carried out ultracentrifugal and viscometric studies of several amylose acetates in methyl acetate solution. Their data, shown in Table VI, were interpreted as supporting the idea of a *helical* configuration in solution.

Foster and Lepow[159] have studied the behavior of amylose solutions in ethylenediamine in a streaming gradient. (Glycerol was added to the solution to increase the viscosity and allow easier orientation of the molecules.) Orientation of the molecules was found, although Peterlin and Samec[160] had previously observed that amylose exhibited no streaming birefringent properties and had concluded the molecule was spherical. Foster and Lepow

TABLE VI

Ultracentrifugal and Viscometric Data for Amylose Acetates in Methyl Acetate Solution

Amylose	$10^{13}S_{20}$	$10^{7}D_{20}$	M	f/f_0	$[\eta]$
Maize	11.2	4.2	187,000	3.70	91
Potato	10.5	6.8	108,000	2.74	56
Tapioca	7.9	8.0	69,000	2.70	43

studied the effect of temperature, concentration, and solvent composition for maize, tapioca, potato, and Easter-lily amyloses, and results were interpreted as suggesting that the orientation effects were predominantly due to the elongation of coiled molecules rather than simple elongation which might occur if a helical configuration were present. Rundle and Baldwin[161] have suggested, from measurements of the dichroism of flow, that the amylose–iodine complex is an oriented straight chain. More recently, Foster and Zuker,[162] have determined the length of the amylose–iodine complex from measurements of streaming dichroism of flow, and have compared this with the length obtained from streaming birefrigence measurements. In the dichroism experiments, use of 0.002 % solutions of amylose in 15 % aqueous pyridine was necessary in order to avoid aggregation. The observed length was less than that expected from the value of $\overline{D.\ P.}$ for the various sub-

(158) A. Peterlin, *J. Polymer Sci.*, **5**, 473 (1950).
(159) J. F. Foster and I. H. Lepow, *J. Am. Chem. Soc.*, **70**, 4169 (1948).
(160) A. Peterlin and M. Samec, *Kolloid-Z.*, **109**, 96 (1944).
(161) R. E. Rundle and R. R. Baldwin, *J. Am. Chem. Soc.*, **65**, 554 (1943).
(162) J. F. Foster and D. Zuker, *J. Phys. Chem.*, **56**, 170, 174 (1952).

fractions studied, although there was a relationship between [η] and length. The addition of amylopectin did not influence the results, and values for whole starch were the same as for amylose. The streaming birefringence measurements on these sub-fractions in both aqueous potassium hydroxide and ethylenediamine (to each of which had been added glycerol), showed lengths which were comparable with those from the dichroism measurements on the corresponding iodine complexes, and it was suggested that, in these solvents, the amylose molecules are helical. The degree of birefringence, measured under fixed conditions, was directly proportional to the limiting viscosity number of the sub-fraction, and the observations also suggested that significant stretching of the molecules may occur at high velocity gradients.

d. Interaction with Iodine.—The characteristic blue color formed when iodine reacts with amylose is well known, but only recently has evidence been obtained of the mechanism responsible. The work of Rundle and his coworkers on the problem is particularly noteworthy. The introduction of potentiometric titrations,[32] as a method of studying the reaction, enabled quantitative data to be obtained. In this method, the measured potential between a bright platinum electrode (in the amylose–iodine solution) and a calomel electrode enabled the equilibrium concentration of free iodine in the mixture to be calculated. A sigmoid type of adsorption isotherm was obtained; the activity of free iodine was constant until amylose had bound from 16 to 20 % of its own weight of iodine (depending on the concentration of iodide ion), after which there was a very slow increase in activity. When the amount of bound iodine was plotted against the total amount of free iodine, the "end point of the titration" (or the "iodine affinity" of the sample) was taken as the point of intersection of the vertical, binding portion of the curve and the linear adsorption curve. It was shown that the percentage of amylose in a starch or an incompletely fractionated amylopectin could be estimated by a comparison of the iodine affinities of these materials with that of pure amylose. This latter value was taken as 18.7 % of bound iodine (for 0.05 *M* iodide). As the affinity of iodine in the complex was a function of molecular weight, it was suggested that the longer amylose molecules bind iodine before shorter ones do.

From measurements of the dichroism of flow of amylose–iodine solutions,[161] and from studies of the optical properties of crystalline amylose platelets and iodine-stained platelets,[163] it was shown, following the suggestion of Hanes, that a *helical configuration* of the amylose in this complex is probable. This was later confirmed by x-ray measurements (see p. 378); the iodine atoms were shown to be situated in the core of helically-oriented amylose molecules.

(163) R. E. Rundle and D. French, *J. Am. Chem. Soc.*, **65**, 558 (1943).

The mechanism of the interaction of iodine with amylose, with which it appears to form a definite inclusion complex and not a solid solution, has been discussed.[164] The very great difference between the molecular extinction coefficient of iodine in starch and in nonpolar solvents suggested that this interaction was of a dipolar nature. It was suggested, in terms of the helical configuration of amylose, that there would be a large dipole along the axis of the helix, due to the summation or reinforcement of the dipoles of the individual D-glucose molecules in the chain. Thus, when an iodine molecule enters the helix, it will be polarized by the permanent dipole of the amylose molecule and be given an induced dipole, and the two will interact through dipolar forces. Succeeding iodine molecules will acquire parallel induced dipoles, and the interactions of these will contribute to the strength of the whole. The magnitude of this induced dipole will be increased with increase in the number of iodine molecules arranged in parallel, and also with the strength of the dipole moment of the amylose molecule, which itself will increase with length. In this manner, the preferential uptake of iodine by long amylose chains was explained by the greater stability of the resultant complex. It was thought that the wavelength of maximum absorption of the iodine complex increases as the number of dipoles increases, so that, for long-chain molecules, the absorption is in the red and the solution appears blue. [The similar blue color between polyvinyl alcohol and iodine was thought to be due to orientation of iodine molecules between the (in this case) parallel chains of polymer molecules.] It was shown from absorption measurements that, in infinitely dilute solutions of iodide ion, there is approximately one iodine molecule per six D-glucose residues.[165] The decrease in bound iodine with increase in iodide was related to the size of the $I_3^{\ominus}$ ion's decreasing the volume available in the center of the helix. Similarly, the binding of fatty acids was explained in terms of the helical model,[166] as the diameter of the helix is approximately equivalent to that of a fatty acid molecule fully extended along the helix axis. The theoretical capacity was only 10 % greater than the experimental value. Dipolar forces were again thought to be responsible for the molecular, inclusion complex, in which the fatty acids were in the holes in the helices rather than in those between the amylose chains.

Stein and Rundle[167] have presented a theory for the dipolar attraction between amylose and iodine in the solid state on the basis of the above

(164) R. E. Rundle, J. F. Foster and R. R. Baldwin, *J. Am. Chem. Soc.*, **66**, 2116 (1944).

(165) R. R. Baldwin, R. S. Bear and R. E. Rundle, *J. Am. Chem. Soc.*, **66**, 111 (1944).

(166) F. F. Mikus, R. M. Hixon and R. E. Rundle, *J. Am. Chem. Soc.*, **68**, 1115 (1946).

(167) R. S. Stein and R. E. Rundle, *J. Chem. Phys.*, **16**, 195 (1948).

helical model, and have discussed the cooperative effects necessary. Dipolar forces were shown to be sufficient to account for the stabilizing energy of the complex, but it was suggested that additional stabilization may result from the resonance effects of a polyiodide chain at high dipolar interaction. It was stressed that modifications of this theory may be necessary in order to account for complex formation in solution, in view of water molecules' altering the dipolar fields and other ions' affecting stability.

Gilbert and Marriott[35] have shown from potentiometric studies that, in the region of very small percentages of bound iodine (less than 0.5%) in 10^{-3} to 10^{-4} *M* iodide, the constitution of the complex is $(3I_2 \cdot 2\ I^{\ominus})$ or $(I_8^{\ominus\ominus})$. This resonating ion was thought to be responsible for the blue color, and other starch products not giving this coloration were thought to be unable to form such a resonating complex. The formation of the complex was shown to be accompanied by the emission of 11.2 kcals./mole of iodine.

Murakami[168] has recently made use of Mulliken's theory[169] of intermolecular charge-transfer spectra in another explanation of the amylose–iodine complex. He showed theoretically that electron transfer between iodine molecules and oxygen atoms in the amylose will give a reasonable explanation of bond number[170] and absorption spectra. The whole system of resonating units in the complex was shown to be successfully approximated to an assemblage of $(I_4^{\ominus\ominus})$ units.

The decrease in amount of bound iodine with increase in iodide-ion concentration is reflected in a decrease in the wave length of maximum absorption of the complex.[165] On the basis of Kuhn's theory for polyenes,[171] this has been related to the breaking of resonating chains of iodine molecules.[172]

Although the dipolar and resonating nature of the interaction of amylose and iodine is well established, Schlamowitz[173] regards the iodine in a starch complex as being in a predominantly non-polar form, and Meyer and Bernfeld[174] refute the helix theory and consider that adsorption of iodine occurs on colloidal micelles in amylose solutions. Most of the experimental facts which Meyer presents can, however, be satisfactorily explained on the helical model.

Rundle and coworkers[165] found that the wave length of maximum absorption (λ_{max}) of the amylose–iodine complex is related to the chain length

(168) H. Murakami, *J. Chem. Phys.*, **22**, 367 (1954).

(169) R. S. Mulliken, *J. Am. Chem. Soc.*, **72**, 600 (1950); **74**, 811 (1954). See also L. E. Orgel, *Quart. Revs.* (London), **8**, 422 (1954).

(170) The number of shared electron pairs in any bond. See L. Pauling, *J. Am. Chem. Soc.*, **69**, 542 (1947).

(171) W. Kuhn, *J. Chem. Phys.*, **17**, 1168 (1949).

(172) S. Ono, S. Tsuchihasdi and T. Kuge, *J. Am. Chem. Soc.*, **75**, 3601 (1953).

(173) M. Schlamowitz, *J. Biol. Chem.*, **190**, 519 (1951).

(174) K. H. Meyer and P. Bernfeld, *Helv. Chim. Acta*, **24**, 389 (1941).

of the amylose. Similar effects were found by Kerr and associates.[175] The relationship between λ_{max} and $\overline{D.\ P.}$ for short, synthetic, linear glucans has been measured by Swanson.[176] Higginbotham[177] found, however, no such relationship for long-chain amyloses.

The results of these measurements, shown in Table VII, are obviously at variance. Some of the differences may be attributable to the different experimental conditions employed. Foster and Paschall[178] have suggested that this change in λ_{max} is related to the state of aggregation of the amylose complex (from studies of the variation of λ_{max} with temperature), and have

TABLE VII

Observed Relationship Between λ_{max} and $\overline{D.\ P.}$ for Amylose-Iodine Complexes

Starch	$\overline{D.\ P.}$	λ_{max} (Å.)	*Molecular extinction coefficient*	*References*
Easter lily	310	6,220	41,400	165
Maize	250	6,180	40,000	165
	480	6,450	—	175
	550	6,200	38,000	177
(crystalline)	175	6,050	40,100	165
(crystalline)	230	6,200	—	175
(crystalline)	300	6,150	37,200	177
Potato	500	6,280	43,000	165
	850	6,500	—	175
	940	6,250	38,500	177
Sago	950	6,250	38,200	177
Tapioca	450	6,250	41,600	165
	1050	6,500–6,550	—	175

suggested[147] that λ_{max} is governed by (1) the average length of the uninterrupted amylose helices, and (2) the degree of crystalline order. Further observations on this effect are necessary before the relationship between λ_{max} and $\overline{D.\ P.}$ can be made unambiguous.

Of the methods available for potentiometrically estimating the amount of iodine bound by amylose, the differential method of Gilbert and Marriott[35] is by far the most satisfactory for accurate work as it eliminates corrections for "reagent blanks." In this method, the amylose solution and control solution form two half-cells connected by a salt-bridge, and the

(175) R. W. Kerr, F. C. Cleveland and W. J. Katzbeck, *J. Am. Chem. Soc.*, 73, 3916 (1951).

(176) Marjorie A. Swanson, *J. Biol. Chem.*, **172,** 825 (1948).

(177) R. S. Higginbotham, *Shirley Inst. Mem.*, **23,** 159 (1949).

(178) J. F. Foster and E. F. Paschall, *J. Am. Chem. Soc.*, **74,** 2105 (1952).

total free iodine concentration in each can be measured directly. The technique has been adapted for routine analyses by Anderson and Greenwood.[36a] The titration of amylose solutions is nevertheless difficult, as the amount bound varies with iodide-ion concentration, temperature, *p*H, and pretreatment. Comparisons between results from different workers is therefore often impossible, and no definite limiting value of iodine affinity for amylose can be quoted. (Results from any given system should, of course, be self-consistent.) Although Higginbotham[44] has suggested that all well-fractionated amyloses bind the same quantity of iodine, this is improbable, as many factors, including molecular weight and its distribution, may influence the results. Schoch[33] has found the iodine affinity to vary between 18.5 and 20.0% (in 0.05 *M* iodide) for several amyloses.

Paschall and Foster[147, 179] have suggested that, in iodine titrations, using Rundle's procedure,[32] the amylose is not in an equilibrium state, as differences between "forward" and "backward" titrations were found. On alkali-aging, disaggregation of aggregated helices was thought to influence results, and retrogradation effects in aged, neutralized solutions were shown by a reduction in binding capacity accompanied by an increase in iodine affinity due to the breakdown of aggregated helices. The effects of variations in titration curves occasioned by differing procedures was shown, but, although these effects may be real, results from comparative measurements should be satisfactory.

3. *Amylopectin and its Derivatives*

a. Degradative Effects in Aqueous Solution.—Schoch[33] and Higginbotham[44] have shown that autoclaving of starches prior to fractionation results in slight degradation of the amylopectin component, as shown by changes in viscosity of the product. Amylopectin also undergoes "mechanical hydrolysis" when subjected (in buffered solution) to the action of a Waring Blendor.[33]

Although degraded by alkali, amylopectin, like amylose, is stable in this solvent in the absence of oxygen. Witnauer, Senti and Stein[180] have studied these effects in the case of potato amylopectin by using light-scattering measurements. These authors also showed that occurrence of aggregation is improbable, as the turbidity was independent of temperature and hydroxyl-ion concentration.

Kerr[181] has suggested that treatment of maize starch granules with 0.1 to 0.15 *N* sulfuric acid results in preferential degradation of the amylopectin,

(179) E. F. Paschall and J. F. Foster, *J. Am. Chem. Soc.*, **75**, 1177 (1953).

(180) L. P. Witnauer, F. R. Senti and M. D. Stein, *J. Chem. Phys.*, **20**, 1978 (1952); *J. Polymer Sci.*, **16**, 1 (1955).

(181) R. W. Kerr, *Die Stärke*, **4**, 39 (1952).

whereas the amylose remains virtually unchanged. (Molecular weights of the acetates of the separated components of acid-treated granules were measured.) A chromatographic analysis of the degradation products has confirmed this preferential hydrolysis of amylopectin.[181a] It should be noted, however, that Meyer and Menzi[20] suggest that, when granules of maize starch are treated with acid, *both* components are degraded by equal proportions.

Swanson and Cori[145] have measured the rate of degradation of amylopectin in 7.7 *N* hydrochloric acid at 30°.

It is convenient to mention at this point experiments involving the disaggregation of starch carried out by Hirst and coworkers. The acid hydrolysis (at *p*H 2) of methylated chestnut[182] and rice starches[88] was followed viscometrically. The initial rate of degradation was very rapid, but, between $\overline{\text{D. P.}}$ of 1000 and 100, the reaction was of the first order. The size of the final product ($\overline{\text{D. P.}} = 100$) was independent of the source of the starch. The energy of activation (20.7 kcal.) suggested that the hydrolysis broke linkages other than hydrogen bonds, and that the lability to acid hydrolysis of the linkages involved was between that of a furanose and pyranose type. The ratio of terminal to nonterminal groups remained constant throughout the hydrolysis, and the results appear to indicate some structural regularity involving about one hundred D-glucose residues. The value for the energy of activation is less than that of 29.0 kcals./mole found for the 4 → 1-α-D bond in starch by Freudenberg and coworkers[183] from experiments involving acid hydrolysis.

b. Molecular Weight and Shape.—Table VIII shows the results of determination of the molecular weight of amylopectins from various sources. It may be seen that chemical methods appear to give exceptionally low values, which cannot be correct. The molecular weight varies with the source of the amylopectin. Of the results obtained from osmotic-pressure measurements, those of Kerr[123] are consistently lower than the others in view of his finding a negative slope of the π/c *versus* c curve near infinite dilution. Kerr believes that higher values are due to aggregation, and suggests that inorganic salts may be the cause, as has been suggested in the

(181a) M. Ulmann, *Makromol. Chem.*, **10**, 147 (1953).

(182) C. E. H. Bawn, E. L. Hirst and G. T. Young, *Trans. Faraday Soc.*, **36**, 880 (1940).

(183) K. Freudenberg, W. Kuhn, W. Dürr, F. Bolz and G. Steinbrunn, *Ber.*, **63**, 1510 (1930).

(184) B. H. Zimm and C. D. Thurmond, *J. Am. Chem. Soc.*, **74**, 111 (1952). This value for the molecular weight was unaffected by temperature, and hence occurrence of aggregation was improbable.

(185) F. E. Horan, *Dissertation, Columbia Univ.*, New York (1944); quoted in Ref. 123.

TABLE VIII

The Results of Molecular-Weight Determinations on Amylopectin and its Derivatives

Starch source	$[\eta]$ M KOH[a]	*Molecular-weight method*[b]	*Derivative*	*Solvent*	*Molecular weight*	$\overline{D.P.}$	*References*
Apple *var.* Newtown Pippin	95	O. P.	acetate	$CHCl_3$	1.2×10^6	4,200	82
Easter lily	126	O. P.	acetate	$CHCl_3$	6×10^6	21,000	103
	126	τ	acetate	CH_3NO_2	420×10^6	1,450,000	184
Hevea brasiliensis, seed	102	O. P.	acetate	$CHCl_3$	1.8×10^6	6,000	26
Maize	—	C.	unsubstituted	H_2O	4.5×10^4	280	31
	124	O. P.	acetate	$CHCl_3$	8×10^6	28,000	103
	135	O. P.	acetate	$CHCl_3$	10×10^6	35,000	103
	125	O. P.	acetate	$CHCl_3$	4.2×10^5	1,450	123
	—	S. & D.	?	?	?	20,000–50,000	152
Passion fruit (*Passiflora edulis*)	—	O. P.	acetate	$CHCl_3$	1.8×10^6	6,000	80
Pea, smooth, *var.* Alaska	157	O. P.	acetate	$CHCl_3$	2×10^6	6,700	47
wrinkled, *var.* Perfection	122	O.P.	acetate	$CHCl_3$	2.5×10^5	870	47
Potato	158	Insoluble	—	—	—	—	123
	—	C.	unsubstituted	H_2O	1.8×10^5	1100	31
	—	S. & D.	?	?	?	6,000	185
Potato I	—	τ	unsubstituted	H_2O	36×10^6	220,000	180
Potato II	—	τ	unsubstituted	H_2O	14×10^6	86,000	180
Potato II	—	τ	acetate	Me_2CO	18×10^6	62,500	180
Sago	82	O. P.	acetate	$CHCl_3$	2×10^6	6,700	103
Tapioca	135	O. P.	acetate	$CHCl_3$	3.7×10^5	1,300	123
	127	O. P.	acetate	$CHCl_3$	6×10^6	21,000	103
Wheat	122	O. P.	acetate	$CHCl_3$	7×10^6	24,000	103
Waxy maize	—	C.	unsubstituted	H_2O	1.8×10^5	1,100	186

[a] Temperature of measurement varies between 22.5–35°C.; [b] O. P. = osmotic-pressure measurements; τ = light-scattering measurements; C. = colorimetric measurements; S. & D. = sedimentation and diffusion measurements.

cellulose field. However, Evans and Spurlin[187] have shown that such ionic effects disappear at infinite dilution, and moreover no analogous effects have been found in light-scattering measurements at low concentrations.[180]

(186) K. H. Meyer and W. Settele, *Helv. Chim. Acta*, **36**, 197 (1953).

(187) E. F. Evans and H. M. Spurlin, *J. Am. Chem. Soc.*, **72**, 4750 (1950).

The density correction is extremely important in such low osmotic-pressure measurements, but has been infrequently applied.

Light-scattering measurements confirm the higher values of molecular weight, but the values of $\bar{M}_w$ are much greater than $\bar{M}_n$. This might suggest that there is a wide molecular-weight distribution, as indeed has been found for potato amylopectin. Witnauer and coworkers[180] have carried out light-scattering investigations on amylopectin isolated by two methods from a *commercial* potato starch. The molecular weight for one sample in water was 36×10^6, whilst that for the other in this solvent was 14×10^6. The corresponding values for the radii of gyration were 1880 Å. and 1090 Å. An *increase* in slope of the Hc/τ *versus* c curves at low concentrations (the converse of the effect described by Kerr) showed the importance of measurements at such dilutions (less than 0.3 %). The polymolecularity of the samples was shown by (1) precipitation with methanol, resulting in a series of fractions whose molecular weight varied from 7×10^6 to 73×10^6 and (2) very broad peaks on the Schlieren diagrams obtained by ultracentrifugation (for example, S_{20} (mean) $= 8.8 \times 10^{-13}$; S_{20} (max.) $= 40.3 \times 10^{-13}$). Turbidimetric measurements showed that, in the absence of oxygen, the amylopectin particles in aqueous solution were unaffected by temperature or by hydroxyl-ion concentration. Addition of potassium chloride to aqueous solutions, however, caused aggregation when the concentration of salt was greater than 1×10^{-3} M. The *partial* acetate (38 to 40 % of acetyl) was soluble in acetone, chloroform, dioxane, nitromethane, and acetonitrile. In all these solvents, the molecular-weight value was comparable with that expected from the unsubstituted amylopectin. If potato amylopectin does in fact possess a molecular weight of many million, reports[44, 123] of the insolubility of the triacetate in chloroform are not surprising.

Further measurements appear necessary before the molecular weights of the amylopectin component of starches can be adequately characterized, and it may well be that light-scattering is the only method which can be satisfactorily applied to these polysaccharides of very high molecular weight. Certainly, it is the only method which enables studies of very dilute solutions to be made with high accuracy, particularly in the case of aqueous solutions.

The viscosities of the triacetates have been measured in only a few instances.[26, 44] A linear relationship between η_{sp}/c and c was found, although Meyer and coworkers[151] have reported this curve to be non-linear.

Kerr[65] has examined the viscometric behavior of three sub-fractions of maize amylopectin obtained by gradual precipitation with methanol, whereas Meyer and Gibbons[72] have found seven sub-fractions of potato amylopectin to be identical with regard to $\overline{\text{D. P.}}$ (colorimetric method).

Few measurements of the shape of the amylopectin component have been reported, although, from viscosity measurements on the β-amylase limit

dextrin (see p. 383), an elongated molecule is possible. This is supported by the approximate linear relationship found between $[\eta]$ and $\overline{D.P.}$[123] The results of streaming birefringence measurements, however, suggest that the particle shape is more spherical.[162]

c. Interaction with Iodine.—Amylopectin forms a characteristic *red* coloration with iodine. Potentiometric titrations[32] showed that the amount of iodine bound by amylopectin in solution is very much smaller than that bound by amylose under the same conditions. Variations in the amount bound by different amylopectins were found, and it was suggested that these were related to differences in length of the repeating unit. Similar differences were found in the absorption spectra.[165] Rundle and coworkers[166] have stressed that this low binding power is inexplicable if complex formation is simply due to hydrogen bonding,[47] and have suggested that the large number of branch points will simply disrupt helix formation and so alter the dipolar forces responsible for complexing. Some external branches in amylopectin were thought to be longer than the average, and Swanson[176] has suggested from a study of absorption spectra of the iodine complex that some of these may be as long as 18 units.

Higginbotham[188] has studied the interaction of amylopectin with iodine over a wide range of iodine and iodide concentrations, and has found that amylopectin can bind as much iodine as can amylose, and that, therefore, a mechanism other than that involving helices must occur. From potentiometric and absorption spectra measurements, he suggested that the iodine is bound partly by a helix mechanism involving chains of I_2 and $I_3^{\ominus}$, and partly by adsorption of single I_2 and $I_3^{\ominus}$ molecules. The amount bound by the helix was thought to vary with the total amount of iodine bound, and no correlation between either potentiometric or colorimetric measurements and the chain length was found.

Gilbert and Hybart,[189] from the results of potentiometric titrations, have suggested that iodine is bound as a complex of one or two iodine molecules together with none, one, or two iodide ions, depending on the concentration of iodide present.

It has not yet been possible to obtain samples of amylopectin which do not show some slight evidence of uptake of iodine by linear material in the early stages of an accurate potentiometric titration. Although this effect is presumably due to contaminating amylose, the presence of some long branches in the amylopectin cannot be excluded. Anderson and Greenwood[190] have shown that in 0.01 M iodide solution, for concentrations of total free iodine less than 1×10^{-5} M, the amount of iodine bound by

(188) R. S. Higginbotham, *Shirley Inst. Mem.*, **23**, 171 (1949).

(189) G. A. Gilbert and F. J. Hybart, unpublished observations; F. J. Hybart, Ph. D. Thesis, University of Birmingham, England (1952).

(190) D. M. W. Anderson and C. T. Greenwood, *Chemistry & Industry*, 642 (1953).

amylopectin is directly proportional to the total free iodine concentration (after corrections have been made for the preferential uptake of any linear material present). From comparisons of the results for different amylopectin and glycogen samples, it was suggested that the uptake of a branched 4 → 1-α-D-glucan is proportional to the length of unit chain. More recent work,[36a] which has involved a study of the abnormal starch-type glucans synthesized by plants and protozoa, suggests that this difference is more fundamentally related to differences in the *degree of multiple branching.* It is possible that this method may be developed so that iodine titrations, in conjunction with estimations of the length of unit chain, may enable some estimate of this to be made. The 36-unit amylopectin isolated from wrinkled-pea starch has been shown to possess abnormal, increased, iodine-binding power.[47] There is also evidence that a considerable number of longer branches may be present in sweet-potato and tapioca amylopectins.[191]

VI. Properties of Starch and Its Components in the Solid State

1. *X-ray Diffraction Studies*

Some molecular orientation is present in starch granules, as they exhibit x-ray diffraction patterns. Efforts have been made to determine the configuration of the starch molecules in the granular state from interpretations of these diffraction data. Exact structural determinations are difficult, however, as, under the usual experimental conditions, the granules only give powder diagrams. Nevertheless, quite useful information can be derived from these. On the basis of the patterns obtained, Katz and coworkers[192] classified starches into two types, namely, those giving an "A"-type pattern (cereal starches) and those giving a "B"-type pattern (tuber starches). (Intermediate "C"-type modifications also occur, but the patterns are usually near one or other of the two extremes.) Another type of pattern ("V"-modification) results when starch is precipitated from aqueous solution with alcohol, but the significance of this will be discussed later. More recently, the fractionation of starch has enabled the diffraction patterns of the components to be studied. Amylose has been found to form films in which molecular orientation can be induced by stretching, so that the product will give a *fiber* pattern. This procedure has enabled more precise data to be obtained, since the pattern of the amylose is very similar to that of the granular starch from which it has been isolated.

a. Granular Starch and Amylose.—The diffraction pattern for a particu-

(191) G. L. Doremus, F. A. Creshaw and F. H. Thurber, *Cereal Chem.*, **28**, 308 (1951)

(192) J. R. Katz and T. van Itallie, *Z. physik. Chem.*, **A150**, 90 (1930); J. R. Katz and J. C. Derkson, *ibid.*, **A165**, 228 (1933); **A167**, 129 (1933).

lar starch granule is not unique. All starches, when retrograded at low temperatures, will give a "B"-type pattern, whilst an "A"-type pattern is produced by retrogradation above 50°. Bear and French[193] have given a careful index of typical "A"- and "B"-type, granular-starch patterns. These authors stressed the experimental conditions necessary to obtain satisfactory patterns (for example, the examination must be carried out under moist conditions), and demonstrated, through variation of temperature, a continuous change from "A"- to "B"-modifications of patterns. The average dimensions of the orthorhombic unit cell were as follows.

modification	*a*	*b*	*c*
"A"	16.1 Å.	9.11 Å.	6.34 Å.
"B"	15.4 Å.	8.87 Å.	6.18 Å.

The greater volume of "A" was taken to be an indication of greater hydration. It was suggested that the unit cell contained two maltose units of a chain of D-glucose units.

Equivalent "A"- and "B"-type patterns are given by either the granules or retrograded starch, or by the starch formed by evaporation of a solution, but when starch is precipitated from solution by alcohol a different modification—the "V"-type—occurs. Bear[194] has examined the various changes which can be induced in this modification by varying the method of preparing the specimen, and he suggested from the data that a *helical* configuration of the molecule in this modification was not improbable.

Although it has been found that the separated amylose component can be readily orientated to yield fiber patterns, amylopectin usually gives poor or amorphous patterns. In the granule, however, amylopectin does exhibit crystallinity, since waxy maize starch gives a diffraction pattern and other waxy starches behave similarly.[193, 195] (This suggests that the branch points in the amylopectin molecule may be in the amorphous part of the granule.)

Rundle and coworkers[196] first investigated the diffraction patterns obtained from stretched films of amylose obtained from maize starch ("B"-modification). The dimensions of the orthorhombic unit cell were as follows.

$$a = 16.0 \text{ Å.}, \quad b \text{ (fiber period)} = 10.6 \text{ Å.}, \quad c = 9.2 \text{ Å.}$$

Eight D-glucose units per unit cell was suggested, and a structure involving *linear* chains of D-glucose units was postulated. It was also stressed that, at the most, only 50 to 60% of the original granule could be crystalline.

(193) R. S. Bear and D. French, *J. Am. Chem. Soc.*, **63**, 2298 (1941).
(194) R. S. Bear, *J. Am. Chem. Soc.*, **64**, 1388 (1942).
(195) See, for example, K. H. Meyer and Maria Fuld, *Helv. Chim. Acta*, **24**, 1404 (1941).
(196) R. E. Rundle, L. Daasch and D. French, *J. Am. Chem. Soc.*, **66**, 130 (1944).

The use of a microtechnique, enabling part of a granule to be irradiated by a narrow pencil of x-rays, has been claimed by Kreger[197] to yield a *fiber* pattern from a large starch grain of the type occurring in the orchid *Phaius grandifolius*. The observations were interpreted as showing an orthorhombic unit cell of dimensions a = 9.0 Å.; b (fiber period) = 10.6 Å.; c = 15.6 Å. for the "B"-modification. The difficulty of forming a repeating unit having a fiber period of 10.6 Å. was stressed, and it was suggested that a spiral of *three* D-glucose units per turn would give this without difficulty. Such a configuration was thought to explain the hexagonal ratio of axes in the basal plane of the unit cell and also the intrinsic birefringent properties of the granule. Since these spirals cannot be packed into the unit cell to give values of density consistent with the observed values, it was suggested that the true cell is orthohexagonal (a = 18 Å.; b = 10.6 Å.). This cell would contain 54 D-glucose units and water molecules. Rundle[198] has criticized Kreger's density data. Spark[199] has suggested that the pattern for amylose in the "V"-modification indicates a rhombic unit cell (a = 13.5 Å.; b = 7.8 Å.; c = 26 Å.), and in the "B"-modification, an orthorhombic unit cell (a = 9.92 Å.; b = 10.6 Å.; c = 15.6 to 16 Å.) containing 8 to 9 D-glucose molecules and some water.

French[200] has collected together unit-cell data for maltose hydrate and some poly-*O*-acylsaccharides in the hope that some packing information might be obtained which could be applied to the problem of starch structure.

It appears that estimates of the dimensions of the unit cell for the "B"-modification of starch (or amylose) are reasonably in agreement, but that structural interpretations of these observations are at variance.

b. Amylose Complexes.—When amylose forms complexes with alcohols, iodine, or fatty acids, the product gives a "V"-type diffraction pattern. Rundle and coworkers[163, 201] have studied the butanol complex and the iodine complex. They analyzed the data from (1) the wet butanol–amylose complex, (2) partially-dried butanol–amylose complex (1 water molecule/D-glucose unit), and (3) the anhydrous amylose. The results of their observations were: (1) orthorhombic unit cell; a = 13.7 Å.; b = 25.6 Å.; c = 7.8 Å. (2) orthorhombic unit cell; a = 13.7 Å.; b = 24.8 Å.; c = 8.05 Å. (3) hexagonal cell; a = 13.0 Å.; c = 8.0 Å. (although this may have been orthorhombic with twice the volume).

(197) D. R. Kreger, *Nature*, **158**, 199 (1946); **160**, 369 (1947); *Biochim. et Biophys. Acta*, **6**, 406 (1951).

(198) R. E. Rundle, *Nature*, **162**, 107 (1948).

(199) L. C. Spark, *Biochim. et Biophys. Acta*, **8**, 101 (1952).

(200) D. French, *Acta Cryst.*, **7**, 136 (1954).

(201) R. E. Rundle and F. C. Edwards, *J. Am. Chem. Soc.*, **65**, 2200 (1943).

When the anhydrous amylose was exposed to iodine vapor, and the iodine complex was investigated, the unit cell was found to be identical with (3). It was therefore suggested that the iodine apparently enters the center of a helix, and a *helical* configuration of amylose in the "V"-modification involving 6 D-glucose units per turn was suggested. (The planes of the D-glucose units were normal to the helix axis.) The external diameter of the helix was 13 Å. and the pitch 8 Å. The hexagonal unit cell was regarded as being formed by the close-packing of such "cylinders" of amylose. Two iodine atoms were suggested per turn of the helix. The true cell was suggested as being orthorhombic and containing two helices. Rundle[202] later carried out a Fourier analysis of the results, and showed the electron-density map of the *c*-projection to be consistent with this proposed helical structure, with iodine atoms in the center. A similar structure was suggested for the amylose–fatty acid complex.[166] Only amylose in the "V"-type modification formed a complex, and this was interpreted as showing that surface adsorption was not occurring. This conclusion has been supported by Katzbeck and Kerr,[203] who showed that even "amorphous" amylose would not form a molecular complex with butanol vapor, fatty acids, and iodine, but that a "V"-modification was necessary, that is, a particular molecular configuration was responsible.

c. Amylose Derivatives.—Whistler and coworkers[204] first showed that stretched films of amylose acetate give typical *fiber* patterns. The fiber period was found to be 18.3 Å., and this behavior was taken as evidence for the linear structure of the molecule.

Senti and Witnauer[205] have reported studies on the fiber diagrams from various alkali-amyloses. Specimens were obtained by deacetylating clamped specimens of amylose acetate with the appropriate alkali. The positions of the alkali ions and the lateral packing of the amylose chains were determined with the aid of Patterson projections. In the "A"- and "B"-modifications, the fiber period was 22.6 Å. (extension of 6 D-glucose units), whilst in the "V"-modification it was 8.0 Å. These authors have also studied in detail the addition compounds of amylose and inorganic salts with special reference to the structure of the potassium bromide–amylose compound.[206] Oriented alkali fibers were treated with the appropriate salt solution. Stoichiometric compounds were formed. The x-ray patterns from these showed that the addition compounds with potassium salts crystallized in

(202) R. E. Rundle, *J. Am. Chem. Soc.*, **69**, 1769 (1947).

(203) W. J. Katzbeck and R. W. Kerr, *J. Am. Chem. Soc.*, **72**, 3208 (1950).

(204) R. L. Whistler and N. C. Schieltz, *J. Am. Chem. Soc.*, **65**, 1436 (1944); R. L. Whistler and G. E. Hilbert, *Ind. Eng. Chem.*, **36**, 796 (1944).

(205) F. R. Senti and L. P. Witnauer, *J. Am. Chem. Soc.*, **68**, 2407 (1946); **70**, 1438 (1948).

(206) F. R. Senti and L. P. Witnauer, *J. Polymer Sci.*, **9**, 115 (1952).

structures possessing either (1) tetragonal or (2) orthorhombic units. Structure (1) was shown by the iodide, bromide, formate, and bicarbonate, and had dimensions a = 10.7 Å.; c (fiber period) = 16.1 Å. The acetate and propionate were isomorphous and possessed structure (2). For the acetate, a = 11.0 Å., b = 18.1 Å., c (fiber period) = 17.9 Å., and for the propionate, a = 11.4 Å., b = 18.0 Å., c (fiber period) = 17.6 Å. The tetragonal structures suggested that amylose chains possess a fourfold screw symmetry with four D-glucose units corresponding to the fiber period of 16.1 Å. Successive residues in the amylose chain must therefore be identical. Patterson projections from the potassium bromide–amylose gave the positions of the inorganic ions in this complex. These positions were confirmed by Fourier projections which, in addition, gave the general outline of the packing of the amylose chains.

VII. Enzymic Studies

Very extensive investigations have been carried out on the enzymes which synthesize and degrade both the starch components, and the general outline of their action pattern is now established. Many reviews of work in this field are available.[207] The determination of the *specific* mode of action of these enzymes, and the use of degradative enzymes for structural determinations, necessitates, however, the use of physical measurements. Only experiments in which physical methods have been involved will be dealt with here. As mentioned previously, inherent difficulties in these investigations are (1) the preparation of *extremely well separated* components for use as substrates (avoiding inadvertent modification[208]), and (2) the preparation of pure enzymes. It is improbable that both of these criteria have been achieved in many investigations to date.

1. *Action of Synthesizing Enzymes*

The synthesis of *linear* 4 → 1-α-D-glucans from D-glucopyranosyl phosphate by the action of phosphorylases has been shown by comparison of results of methylation and end-group assay and viscosity determination,[209] and by potentiometric, iodine titrations[32] on the product. The chain length of the enzymic product (100 to 200 D-glucose units) is less than that of the natural component. Whether this is due to impure enzymes cannot yet

(207) K. Myrbäck, *Advances in Carbohydrate Chem.*, **3**, 251 (1948); Mary L. Caldwell and Mildred Adams, *ibid.*, **5**, 229 (1950); D. J. Manners, *Ann. Repts. on Progr. Chem.* (Chem. Soc. London), **50**, 288 (1954); S. A. Barker and E. J. Bourne, *Quart. Revs.* (London), **7**, 56 (1953).

(208) See, for example, Barbara Illingworth, J. Larner and Gerty T. Cori, *J. Biol. Chem.*, **199**, 631 (1952); also Ref. 43.

(209) W. Z. Hassid and R. M. McCready, *J. Am. Chem. Soc.*, **63**, 2171 (1941).

be decided, but it is hoped that further investigations will be carried out to study the mode of action of the enzyme.

The formation of a *branched product* from amylose, by the action of the Q-enzyme isolated by Peat, has been studied by Nussenbaum and Hassid.[210] These authors presented evidence that the synthetic product is highly branched, as it was shown to possess a chain length (from periodate and enzymic assay) of 20 to 21 D-glucose units. The osmotically-determined molecular weight of the acetylated product from 24-hour, enzymic incubation was 97,000 ($\overline{\text{D. P.}} = 330$). The original amylose had a $\overline{\text{D. P.}}$ of 600, and hence some decrease in size had also occurred. An ultracentrifugal examination of the product showed a single product ($S_{20} = 3.1$), which was polymolecular. The difficulty of obtaining pure enzymes is illustrated by a similar experiment by Gilbert, Greenwood and Patrick,[211] in which potato amylose was acted on by *crystalline* Q-enzyme. Samples of polysaccharide were removed throughout the course of the enzyme action, acetylated, and examined in chloroform solution by osmometry and viscosity. A slight breakdown occurred, which was shown, by preferential inactivation and by a comparison of different enzyme preparations, to be due to amylase impurity and not to Q-enzyme action. Although difficult, more rigorous purification of this enzyme is necessary before its mode of action can be established.

These synthetic linear and branched molecules may be important as *type polymers*, particularly if the interconversion of amylose to amylopectin is intramolecular, in which case the initial molecular weight and molecular-weight distribution would be retained. There is the possibility that the *in vitro* synthesis may even result in a truly three-dimensional structure, as distinct from that of the natural component.

2. *Action of Degradative Enzymes*

A large number of starch-degrading enzymes (amylases) exist. They can be conveniently classified as (1) those giving maltose (β-amylases), (2) those giving more than one sugar (α-amylases), (3) those giving D-glucose (amyloglucosidases), and also (4) a group of debranching enzymes.

β-Amylase operates exclusively on nonreducing, terminal units in amylose or on the branches in amylopectin, to produce maltose directly, and its hydrolytic action on 4 $\rightarrow$ 1-α-D linkages is stopped by any branch points. Enzyme action is greatly impeded by secondary valence forces, as retrogradation, for example, is accompanied by an increased resistance to β-amylolysis.[19] The enzyme can be crystallized relatively easily. The mode

(210) S. Nussenbaum and W. Z. Hassid, *J. Biol. Chem.*, **190**, 673 (1950).

(211) G. A. Gilbert, C. T. Greenwood and A. D. Patrick, *Intern. Congr. Biochem., Abstr. of Communs., 2nd Congr., Paris*, 1952, p. 241.

of degradative action on amylose has been in dispute.[212] The mechanism may be of a "single-chain" type (in which the enzyme attacks one amylose molecule and degrades it completely before attacking another), or a "multi-chain" type (in which all amylose molecules are shortened simultaneously), or any intermediate between the two. Kerr and Gehman[213] have reviewed the evidence for each of these mechanisms, but appear to have shown conclusively (in the case of recrystallized β-amylase acting on highly purified, maize amylose) that the action is, in its initial stages, a "single-chain" mechanism. The polymeric product isolated at about 50 % conversion to maltose was found to possess virtually the same iodine affinity, limiting viscosity number, and $\overline{\text{D. P.}}$ as the original amylose (see Table IX). Kerr has also shown that the rate of production of maltose from maize amyloses of varying $\overline{\text{D. P.}}$ was dependent on molarity and not on the concentration—a result to be expected if β-amylase acts in a terminal-wise manner.

Schoch and coworkers[33] have examined the limiting viscosity number of

TABLE IX

Properties of Original Amylose and High-Polymer Residue[213]

Property	*Original amylose*	*Recovered residue*
$\overline{\text{D. P.}}$ (from osmotic pressure of acetate in $CHCl_3$)	235	235
Limiting viscosity number (M KOH at 35°)	46	48
Iodine bound, %	20.1	20.4

the polymer residue from the action of β-amylase on maize amylose. The values of $[\eta]$ for 0 %, 41 %, and 61 % conversion to maltose were 1.23, 1.39, and 1.38, respectively, and they suggested that the shorter amylose molecules were attacked first and the longer ones were left. This result also substantiates the "single-chain" mechanism.

Obviously, more work on this subject is necessary, but the problem is complicated by the fact that some authors have reported that the conversion of amylose to maltose by the action of β-amylase is incomplete. Limits of from 70 to 97 % have been recorded. Apparently, variations depend on the actual $\overline{\text{D. P.}}$ of the amylose and on the concentration of enzyme. Meyer and coworkers[19, 214] maintain that these lower limits may be due to retrogradation phenomena, which is not unreasonable in view of the fact that

(212) Compare: Marjorie A. Swanson, *J. Biol. Chem.*, **172**, 825 (1948); F. C. Cleveland and R. W. Kerr, *Cereal Chem.*, **25**, 133 (1948); R. H. Hopkins and B. Jelinek, *Nature*, **164**, 955 (1949); E. J. Bourne and W. J. Whelan, *ibid.*, **166**, 258 (1950); R. W. Kerr and F. C. Cleveland, *J. Am. Chem. Soc.*, **73**, 2421 (1951); R. H. Hopkins and B. Jelinek, *Biochem. J.* (London), **56**, 136 (1954); R. Bird and R. H. Hopkins, *ibid.*, **56**, 140 (1954).

(213) R. W. Kerr and H. Gehman, *Die Stärke*, **3**, 271 (1951).

(214) K. H. Meyer and W. F. Gonnon, *Helv. Chim. Acta*, **34**, 308 (1951).

enzymic digests are normally at *p*H 4 to 5, in which range amylose is unstable. Peat, Pirt and Whelan[215] maintain, however, that this limit is due to the presence of anomalous β-D-linkages in the amylose as, after the addition of Z-enzyme (a β-D-glucosidase) to the limit dextrin, the addition of more β-amylase completes hydrolysis to maltose. The possibility of anomalous groups or linkages cannot, however, be overlooked.[43] Clearly, a full examination of the "amylose limit dextrin," including its molecular size and shape, is essential.

Kerr[123] has found the limiting viscosity number (in 1 *M* potassium hydroxide) of the β-limit dextrin of maize amylopectin ($\overline{\text{D. P.}}$ = 800) to be essentially the same as that of the parent amylopectin—a result substantiated by Schoch and coworkers[33] from similar measurements on waxy-maize starch limit dextrin. Schoch has interpreted this as showing that, since $[\eta]$ is a function of the axial ratio, the consistency of this value must indicate that the amylopectin possesses a highly branched, globular structure. However, it is more probable that the $[\eta]$ would not change much after β-amylolysis if the structure were similar to that suggested by Haworth and Hirst. In this instance, the model is essentially elongated, and the result of enzymic degradation would be merely the removal of the short side-chains, leaving the general shape of the molecule little altered. The great difference between the viscometric behavior of glycogen and amylopectin,[26] as also between the two β-limit dextrins,[97] suggests, moreover, that amylopectin does not possess a randomly-branched, globular structure as does glycogen. Further experiments on the size and shape of the amylopectin β-limit dextrin are necessary.

Since the limit dextrin differs from the original amylopectin only in that the external chains consist of two or three D-glucose units,[101, 216] the original length of external chains can be calculated from the β-amylolysis limit and the average length of unit chain. Low molecular-weight subfractions of waxy maize starch and tapioca amylopectin examined by Meyer and Settele[186] in this manner were found to possess varying external chain-lengths.

The mode of action of *α-amylases* depends on the source from which the enzyme was isolated and on the substrate, and the data available present a bewildering array of facts difficult to correlate.

Kerr, Cleveland and Katzbeck[217] have shown that the *amyloglucosidase* from *Aspergillus niger* hydrolyzes amyloses to D-glucose by a single-chain mechanism, as equimolar solutions of several maize amyloses (with $\overline{\text{D. P.}}$ values from 135 to 480) gave the same rate of D-glucose production. The action of this enzyme, together with that of β-amylase, has been used[154] to

(215) S. Peat, S. J. Pirt and W. J. Whelan, *J. Chem. Soc.*, 705, 714 (1952).
(216) B. Illingworth, J. Larner and Gerty T. Cori, *J. Biol. Chem.*, **199**, 631 (1952).
(217) R. W. Kerr, F. C. Cleveland and W. J. Katzbeck, *J. Am. Chem. Soc.*, **73**, 3916 (1951).

determine the degree of branching in amyloses by measuring the rate of sugar production. If each enzyme acts by a truly "single-chain" mechanism, then the rate of sugar production will be proportional to the number of terminal groups present. Equimolar solutions of the amyloses were hydrolyzed and the rates of sugar production were compared with that determined for a sample of crystalline maize amylose, which was shown by comparison of molecular weight and periodate-oxidation results to be linear. The rates for other maize amyloses were substantially the same as for this standard, indicating that these molecules were essentially linear, whereas those for potato and tapioca amylose were approximately double and triple, respectively. It was suggested that there was an average of one to two branches per potato-amylose molecule and two to three branches per tapioca-amylose molecule. The effect of any contaminating amylopectin was shown not to affect the rates of hydrolysis. Variations in the degree of branching found by different authors for amyloses from the same source were thought to be due to the use of different fractionation procedures, resulting in the precipitation of varying amounts of "intermediate-type" polymers.

Amylo-1 → 6-glucosidase obtained by Cori and Larner[218] from rabbit muscles, and *R-enzyme* isolated by Hobson, Whelan and Peat[219] from potatoes and broad beans, are typical debranching enzymes, which will hydrolyze the 6 → 1-α-D-glucosidic linkage rather than the normal 4 → 1-α-D linkage. These enzymes will therefore be particularly important in determinations of the fine structure of amylopectin, if they can be sufficiently well purified.

3. *Determination of Fine Structure*

Comparison of the size and shape of products resulting from the action of highly purified enzymes on the starch components with those of the original polysaccharides will often give valuable information, although, as outlined earlier, further investigations are necessary. The use of more than one degradative enzyme can, however, often lead to information regarding the finer details of molecular structure.

The only example of this technique applied to the amylose component is that already described, of the action of Z-enzyme on the β-limit dextrin. In the case of amylopectin, enzymic methods enable a distinction to be made between the proposed "laminated" and highly ramified structures (I and III, in Fig. 1, page 352). The method used by Peat and coworkers[101] involves the successive action of β-amylase and R-enzyme on waxy maize starch. β-Amylolysis will degrade A-chains down to two or three units from the 6 → 1-α-D interchain linkages. These latter linkages will protect the

(218) Gerty T. Cori and J. Larner, *J. Biol. Chem.*, **188**, 17 (1951).
(219) P. N. Hobson, W. J. Whelan and S. Peat, *J. Chem. Soc.*, 1451 (1951).

B-chains until they are acted on by R-enzyme, when maltose or maltotriose will be produced from the residual A-chain, and linear dextrins from the B-chains. The amount of maltose or maltotriose liberated on treating the β-limit dextrin with R-enzyme will be a measure of the number of A-chains in the molecule, and from these data, the ratio of A:B chains in the molecule can be calculated.[220] Peat concluded that multiple branching is an intrinsic part of the amylopectin structure, as the observed yield of these sugars was greater than expected for a singly-branched structure. It should be noted that glycogen has been shown by similar enzymic methods to possess a truly random structure.[221]

Such enzymic determinations of fine structure are extremely valuable, but the experimental technique is difficult and a quantitative interpretation of the data has to be made with caution.

VIII. Conclusions

Although a wide variety of starches have been studied, in very few instances have the size and shape of the components been adequately characterized. The labile nature of these materials may account for much of the variation in the reported data, and precautions must be taken to avoid inadvertent degradation during isolation. Further work is also necessary to determine uniquely the fine structure of both components, and the possibility of "intermediate fractions" cannot be ignored. Although there is, at the moment, little evidence for existence of these, it seems improbable that *only two* chemically distinguishable entities should exist in the granule. Little work on the molecular-weight distribution of either component has been carried out.

Indeed, notwithstanding extensive investigations on the starch components, many problems requiring the application of physical methods remain incompletely solved.

Addendum

The Size and Shape of Some Polysaccharide Molecules

I. Introduction

The results of measurements of the size and shape of polysaccharides (with the exception of starch and cellulose) have previously been reviewed to the end of 1951.[107] This addendum covers the field to about the end of 1955.[222] Although certain developments in methods have been dealt with earlier in this Chapter, the following are of general importance.

(220) E. L. Hirst and D. J. Manners, *Chemistry & Industry*, 224 (1954).

(221) J. Larner, B. Illingworth, Gerty T. Cori and C. F. Cori, *J. Biol. Chem.*, **199**, 641 (1952).

(222) It should be noted that the term *polydisperse* is now used to describe polymer

The determination of molecular weights of less than 20,000 (a range which is common in the hemicellulose field) is difficult. *Isothermal distillation*[223] is one suitable method. The development of polyvinyl alcohol membranes, which are claimed[224] to be semipermeable down to $\bar{M}_n \approx 2{,}000$, is therefore of great interest. If these membranes are satisfactory, many problems of osmometry in this low molecular-weight range will be solved. The scope of dynamic ultracentrifugal experiments has also been extended to include these low molecular weights by the development of the *synthetic-boundary cell*.[225] Moreover, it has been shown[226] that Archibald's procedure,[227] can be satisfactorily used to evaluate ordinary sedimentation experiments with materials having $\bar{M}_w < 10{,}000$.

Difficulties in estimating the concentration of components in polydisperse sedimenting systems have been investigated,[228] and the non-ideality correction in sedimentation equilibrium experiments analyzed.[229] In viscosity measurements, corrections for kinetic energy and shear should be applied where necessary. When dealing with polysaccharides containing ionizable groups, electroviscous effects[230] have to be considered. Such charge effects also influence light-scattering experiments.[231]

It will be seen that, although more work has been reported, in no case has the size and shape of a polysaccharide been unequivocally determined.

II. The Molecular Weights of Polysaccharides Containing One Type of Structural Unit

1. *Xylans*

Few further measurements have been reported. The essentially unbranched structure of a xylan from wheat straw[232] and of hemicellulose-A[233]

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systems containing more than one component, whilst *polymolecular* denotes a homogeneous polymer having a variation in molecular weight (see Ref. 2).

(223) G. Gee, *Trans. Faraday Soc.*, **36**, 1164 (1940); R. L. Parette, *J. Polymer Sci.*, **15**, 447 (1955).

(224) H. T. Hookway and R. Townsend, *J. Chem. Soc.*, 3190 (1952).

(225) G. Kegeles, *J. Am. Chem. Soc.*, **74**, 5532 (1952); E. G. Pickels, W. F. Harrington and H. K. Schachman, *Proc. Natl. Acad. Sci. U. S.*, **38**, 943 (1952); H. K. Schachman and W. F. Harrington, *J. Polymer Sci.*, **12**, 379 (1954).

(226) R. Brown, D. Kritchevsky and M. Davies, *J. Am. Chem. Soc.*, **76**, 3342 (1954).

(227) W. J. Archibald, *J. Phys. & Colloid Chem.*, **51**, 1204 (1947).

(228) J. R. Cann, *J. Am. Chem. Soc.*, **75**, 4218 (1953); W. F. Harrington and H. K. Schachman, *ibid.*, **75**, 3533 (1953); R. Trautman, V. Schumaker, W. F. Harrington and H. K. Schachman, *J. Chem. Phys.*, **22**, 555 (1954).

(229) K. E. van Holde and J. W. Williams, *J. Polymer Sci.*, **11**, 243 (1953).

(230) Compare: R. M. Fuoss, *J. Polymer Sci.*, **3**, 603 (1948); **4**, 96 (1949); U. P. Strauss and E. H. Smith, *J. Am. Chem. Soc.*, **75**, 6186 (1953).

(231) Compare: P. Doty and R. F. Steiner, *J. Chem. Phys.*, **20**, 85 (1952); U. P. Strauss, E. H. Smith and P. L. Wineman, *J. Am. Chem. Soc.*, **75**, 3925 (1953).

(232) G. O. Aspinall and R. S. Mahomed, *J. Chem. Soc.*, 1931 (1954).

from beech wood has been established. Values of 50 and 70, respectively, were obtained for the degrees of polymerization ($\overline{D.\ P.}$) of the methylated derivatives from the results of isothermal distillations carried out by Greenwood. The effect of alkali on the $\overline{D.\ P.}$ of beech hemicelluloses has been studied viscometrically.[234] A $\overline{D.\ P.}$ of 175 ± 10 for beech xylan has been reported from visual measurements of the light scattered from solutions of the mixed acetyl benzoyl ester in dioxane.[234a]

2. *Glucans (with the Exception of Starch and Cellulose)*

a. Glycogen.—Recent work suggests that some glycogens are polydisperse. From sedimentation measurements on samples of normal-liver glycogen, Polglase, Brown and Smith[235] found evidence for two polymolecular components. Values of S_{20} were (60 to 100) × 10^{-13} for the main component and (150 to 300) × 10^{-13} for the minor one. Glycogen from the liver of a case of *von Gierke's disease* was reported to contain only the lighter component. However, a sample of this glycogen examined by Greenwood was polydisperse in the ultracentrifuge.[236] Many other glycogen samples have also been found to be polydisperse.[237]

Values of molecular weight (uncorrected for dissymmetry) have been obtained by Harrap and Manners[238] from light-scattering investigations, as follows: rabbit liver, 6.8 × 10^6; rabbit muscle, 2.8 × 10^6; cat liver, 10.0 × 10^6; fetal sheep liver, 14.8 × 10^6; *Tetrahymena pyriformis*, 9.8 × 10^6; and *Ascaris lumbricoides*, 8.8 × 10^6.

The effect of alkali treatment on molecular weight (compare with the case of the starch components) has been studied; treating a 5% solution of rabbit-liver glycogen in 2 *N* sodium hydroxide, for 90 minutes at 100°, decreased the sedimentation constant (S_{20} × 10^{13}) from 86 to 57 (that is, by 34%).[237]

b. Laminarin.—Sedimentation and diffusion measurements on two laminarins (*d* and *c*), from *Laminaria digitata* (*d*) and *Laminaria cloustoni* (*c*), have been carried out by Cook and coworkers.[239] Values of S_{20} and D_{20} were found to be dependent on the ionic strength of the buffer used, except in the case of borate. Both samples were polymolecular, and values of 5,300 and 4,500 were given for the molecular weight of *laminarin d* and *laminarin c*, respectively. The difference in solubility of the two forms of laminarin

(233) G. O. Aspinall, E. L. Hirst and R. S. Mahomed, *J. Chem. Soc.*, 1934 (1954).
(234) V. Prey, E. Waldman and W. Krzandalsky, *Monatsh.*, **84**, 888 (1953).
(234a) M. Horio, R. Inamura and H. Inagaki, *Tappi*, **38**, 216 (1955).
(235) W. J. Polglase, D. M. Brown and E. L. Smith, *J. Biol. Chem.*, **199**, 105 (1953).
(236) D. J. Manners, *J. Chem. Soc.*, 3527 (1954).
(237) C. T. Greenwood and D. J. Manners, unpublished observations.
(238) B. S. Harrap and D. J. Manners, *Nature*, **170**, 419 (1952).
(239) M. H. G. Friedlaender, W. H. Cook and W. G. Martin, *Biochim. et Biophys. Acta*, **14**, 136 (1954).

(see Ref. 107) was thought to be related to some structural difference. In addition, methylation was shown to be accompanied by a decrease in molecular weight.

c. Bacterial Dextrans.—Investigations of the size and shape of the important dextrans from *Leuconostoc mesenteroides* have been reported.[240] A study of acid-hydrolyzed dextrans has been performed by Wales, Marshall and Weissberg.[241] In the range $20{,}000 < M < 250{,}000$, the relationship $[\eta] = 10^{-3} M^{0.5}$ was suggested for the degraded dextrans in aqueous solution at 25°C. This relationship has been confirmed[242] from light-scattering measurements.

In the range of molecular weight of 60,000 to 100,000, the relationship

$$\log \bar{M}_w = 2.5714 \log [\eta] + 6.3998$$

has been suggested from light-scattering and viscosity measurements.[242a]

Arond and Frank[243] have fractionated a dextran by precipitation with methanol. Molecular weights from light-scattering measurements ranged from (12 to 600) $\times 10^6$. The molecular inhomogeneity of the fractions was estimated. Flory's theory[130] did not satisfactorily explain the viscometric data, and this was thought to be attributable to the highly branched nature of the polysaccharide and the inhomogeneity of the fractions.

Ogston and Woods,[244] from calculations based on the data of Ingelman and Halling,[107] have concluded that dextran molecules are highly hydrated and *not* very asymmetric. These conclusions were extended by sedimentation measurements on samples of fractionated dextrans.[245] All the samples were found to be polymolecular and the distribution of sedimentation constant in each was determined using the method of Baldwin.[135] Calculations of the mean dimensions of the particles in solution again suggested they were highly hydrated and not very aspherical. These calculations and those of molecular weight from S_{20} and $[\eta]$ (see Table X) were made using the method of Ogston.[109]

(240) A complete characterization and classification of dextrans from 96 strains of bacteria has recently been carried out. See, Allene Jeanes, W. C. Haynes, C. A. Wilham, J. C. Rankin, E. H. Melvin, Marjorie J. Austin, J. E. Cluskey, B. E. Fisher, H. M. Tsuchiya and C. E. Rist, *J. Am. Chem. Soc.*, **76,** 5041 (1954).

(241) M. Wales, P. A. Marshall and S. G. Weissberg, *J. Polymer Sci.*, **10,** 229 (1953).

(242) J. A. Riddick, E. E. Toops, R. L. Wieman and R. H. Cundiff, *Anal. Chem.*, **26,** 1149 (1954); F. R. Senti, N. N. Hellman, N. H. Ludwig, G. E. Babcock, R. Tobin, C. A. Class and B. L. Lamberts, *J. Polymer Sci.*, **17,** 527 (1955).

(242a) S. V. R. Mastrangelo, Barbara Clay, M. M. Fishman, A. G. Hagan, A. Lazrus and W. Zagar, *Anal. Chem.*, **27,** 262 (1955).

(243) L. H. Arond and H. P. Frank, *J. Phys. Chem.*, **58,** 953 (1954).

(244) A. G. Ogston and E. F. Woods, *Nature*, **171,** 221 (1953).

(245) A. G. Ogston and E. F. Woods, *Trans. Faraday Soc.*, **50,** 635 (1954).

3. *Galactans*

a. Carrageenin.—This water-soluble polysaccharide can be isolated[246] from certain seaweeds, for example, *Chondrus crispus*. It is a sulfated galactan, in which linkages occur through C1 and C3 and sulfate ester groups are attached to C4. Cook and coworkers[247] have investigated some samples possessing a high viscosity. All were electrophoretically homogeneous, although, in the ultracentrifuge, two components were present. (The minor, high molecular-weight component varied between 0 and 12% as the viscosity of the sample increased.) The sedimentation constant was dependent on concentration. The values obtained are given in Table XI.

The components appearing on ultracentrifuging have been separated by fractional precipitation with potassium chloride,[248] and the physical proper-

TABLE X

Molecular Data for Some Dextran Fractions[245]

Sample	$10^{13}S_{20}$	$[\eta]$	$10^{-4}M$	*Axial ratio*[a]
A	9.6	64	46.0	4.5
B	5.47	37	15.0	5.1
C	2.02	14	2.0	6.9
49,023	3.47	33	6.5	7.9
40,000	3.04	20	4.4	5.9

[a] Compare with values of 17 to 110 found by Ingelman and Halling,[107] neglecting hydration. Values of (l/d) of 4.5 to 7.9 correspond to values in (f/f_0) of about 1.2 to 1.4.

ties of the two (termed κ- and λ-carrageenin) have been studied.[249] Although ultracentrifugal examination of the whole material indicated only 18% of the λ-component, precipitation resulted in a yield of 45%. Large boundary-anomaly effects therefore occurred. The sedimentation constant for each of the separated components was very dependent on concentration, and estimates of the molecular weight from Flory's theory[130] indicated values for the κ-component of (1.8 to 3.2) × 10^5, and values for the λ-component of (4 to 7) × 10^5. Each fraction was heterogeneous. It was suggested that two different polysaccharides were present in the original carrageenin.

A molecular weight of 120,000 for the sodium salt of a sample of carra-

(246) T. Mori, *Advances in Carbohydrate Chem.*, **8**, 315 (1953).

(247) W. H. Cook, R. C. Rose and J. R. Colvin, *Biochim. et Biophys. Acta*, **8**, 595 (1952).

(248) D. B. Smith and W. H. Cook, *Arch. Biochem. and Biophys.*, **45**, 232 (1953).

(249) D. B. Smith, W. H. Cook and J. L. Neal, *Arch. Biochem. and Biophys.*, **53**, 192 (1954).

geenin has been determined from osmotic-pressure measurements,[250] whereas a value of 1.7×10^6 was found by Goring[134] by light-scattering. The angular distribution of the scattered light suggested that the molecule was a stiff rod of length about 3700 Å.

The variations in the value of molecular weight may be due to the polymolecularity of the samples and to inadvertent degradation during isolation.

4. *Fructans*

From sedimentation and diffusion measurements, Ogston has determined the molecular weight of the fructans from both leafy cocksfoot grass (*Dactylis glomerata*) and Italian rye grass (*Lolium italicum*) to be 5,500.[251] Both polysaccharides were polymolecular, and the data indicated a singly-branched structure for each.

TABLE XI

Molecular Data for Carrageenin[250]

Sample	η_{sp}/c^a	$10^{13}S_{20}$	10^7D_{20}	$10^{-5}M^b$	f/f_0^c
L1	3.4	3.63	1.41	1.2	4.0
C1	4.3	3.24	1.39	1.1	4.3
C2	6.1	3.94	0.95	2.0	5.2
C3[d]	11.2	6.83	0.61	5.3	5.8

[a] c, 0.1 g./100 ml. in 0.05 M NaCl. [b] V, 0.5. [c] Assuming 50% hydration. [d] The molecular weight was 250,000 from osmotic-pressure measurements.

5. *Polyuronides*

a. Pectic Materials.—Osmotic-pressure measurements carried out by Pals and Hermans[252] on an unsubstituted, commercial sample of pectin gave a molecular weight of 460,000. Pippen, Schulz and Owens[253] recommend Vollert's method of using diazomethane at −20°C. (see Ref. 107) to avoid degradation during esterification. The viscosity of the fully esterified derivative was independent of *p*H. Although the limiting viscosity number decreased with increase in esterification, the molecular weight (determined from osmotic-pressure measurements) was *unchanged*. The importance of using osmotic methods to follow degradation was emphasized.

b. Alginic Acid.—Cook and Smith[254] have carried out sedimentation, diffusion, and viscosity measurements on five unfractionated samples of

(250) C. R. Masson and G. W. Caines, *Can. J. Chem.*, **32**, 57 (1954).
(251) D. J. Bell and Anne Palmer, *J. Chem. Soc.*, 3763 (1952).
(252) D. T. F. Pals and J. J. Hermans, *Rec. trav. chim.*, **71**, 458 (1952).
(253) E. L. Pippen, T. H. Schulz and H. S. Owens, *J. Colloid Sci.*, **8**, 97 (1953).
(254) W. H. Cook and D. B. Smith, *Can. J. Biochem. and Physiol.*, **32**, 227 (1954).

sodium alginate. The sedimentation constant was very dependent on concentration, and various methods were used to determine the limiting value of S_{20}. The diffusion constants were believed to be overestimated. Table XII shows the results obtained. Various methods of evaluating the data were presented, and use was made of Flory's theory[130] to suggest that the values of molecular weight of the samples ranged between 47,000 and 370,000. The relationship $[\eta] = 7.5 \times 10^{-5} \times M$ was given, and the viscosity data also indicated that the molecules had a high extension ratio.

III. The Molecular Weights of Polysaccharides Containing More Than One Type of Structural Unit

1. *Plant Gums and Mucilages*

a. Gum Arabic.—The molecular weight of a laboratory-prepared sample of gum arabic has been found to be $(1.0 \pm 0.05) \times 10^6$ from light-scatter-

Table XII

Molecular Data for Sodium Alginate Samples[254]

Sample[a]	1	2	3	4	5
$[\eta]$	310	505	878	1610	1750
$10^{13}S_{20}$[b]	2.08–2.31	2.47–2.69	2.78–3.27	3.30–4.20	3.75–5.15
$10^{7}D_{20}$	2.75	1.66	1.35	1.16	1.10

[a] Measurements in 0.1 *M* NaCl buffered to *p*H 6.6 with phosphate; $V = 0.44$.
[b] Range shows variation due to method of evaluating the sedimentation results.

ing investigations.[255] It was suggested that the molecule behaves in solution as a stiff coil, varying in extension from 1050 Å. at zero charge to 2400 Å. at maximum charge. This value is considerably higher than any previously reported (see Ref. 107).

b. Tororo-aoi (Abelmoschus Manihot, *Medic.*) *Mucilage.*—This mucilage is used extensively in papermaking in Japan. Machida and Uchino[256] have shown it to be a polyrhamnogalacturonide possessing typical polyelectrolyte behavior. The $\overline{\text{D. P.}}$ of the nitrate, in acetone, was about 390 (from viscosity measurements using Staudinger's law).

c. Arabinogalactans.—The water-soluble arabinogalactan from Douglas fir (*Pseudotsuga taxiflora*) has been examined by Wise and associates.[257] It possessed a D. P. of 340 from osmotic pressure measurements on the ace-

(255) A. Veiss and D. N. Eggenberger, *J. Am. Chem. Soc.*, **76**, 1560 (1954).
(256) S. Machida and N. Uchino, *Bull. Fac. Textile Fibers, Kyoto Univ. Ind. Arts and Textile Fibers*, **1**, 116 (1954).
(257) J. O. Thomson, J. J. Belcher and L. E. Wise, *Tappi*, **36**, 319 (1953).

tate. That from Black spruce (*Picea mariana*) was similarly found[258] to possess a $\overline{D.P.}$ of 330.

2. *Mucopolysaccharides*

a. Microbiological Polysaccharides.—The capsular polysaccharide from an amylolytic, sheep-rumen streptococcus[259] has been examined by Greenwood.[260] The material was homogeneous in the ultracentrifuge (S_{20} = 3.25×10^{-13}) and possessed a limiting viscosity number of 3.75. This high value indicated an extended shape in solution ($f/f_0 = 3.5$), and the molecular weight calculated using this value was 90,000.

The specific polysaccharide of the dominant "O" somatic antigen of *Shigella dysenteriae* has been shown to be electrophoretically and ultracentrifugally homogeneous, and to possess a molecular weight of 26,000 from sedimentation and diffusion measurements.[260a]

b. Blood-group Polysaccharides.—Kekwick[261] has examined the Lewis blood-group substance[262] and the blood-group H substance.[263] Both were electrophoretically homogeneous. The sedimentation constant varied with concentration. For the Lewis factor, the molecular weight was 270,000 ($S_{20} = 5.44 \times 10^{-13}$; $D_{20} = 1.37 \times 10^{-7}$; $V = 0.643$) and the frictional ratio (f/f_0) indicated high asymmetry or hydration. The H factor had a higher molecular weight of 320,000 ($S_{20} = 5.21 \times 10^{-13}$; $D_{20} = 1.21 \times 10^{-7}$; $V = 0.636$), and also a larger frictional ratio ($f/f_0 = 4.2$). Casprey[264] has examined two glycopolysaccharides (B and B′) possessing blood-group B character.[265] Both components were electrophoretically homogeneous. Material B′ was homogeneous in sedimentation experiments, whereas B contained a small, polymolecular, heavier component. Sedimentation and diffusion constants were dependent on the concentration ($V = 0.604$ for B' and 0.600 for B). Values of molecular weight were B′ = 460,000 and B = 1,800,000 (approx.) with corresponding values for (f/f_0) of 3.9 and 5.7. Both were polymolecular and probably highly asymmetrical. These values are higher than those for other blood-group substances.

c. Hyaluronic Acid.—Jensen[266] has claimed the isolation of samples of

(258) J. O. Thomson, J. J. Belcher and L. E. Wise, *Tappi*, **36**, 541 (1953).
(259) P. N. Hobson and Margaret J. Macpherson, *Biochem. J.* (London), **57**, 145 (1954).
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(263) E. F. Annison and W. T. J. Morgan, *Biochem. J.* (London), **52**, 247 (1952).
(264) E. A. Casprey, *Biochem. J.* (London), **57**, 295 (1954).
(265) R. A. Gibbons and W. T. J. Morgan, *Biochem. J.* (London), **57**, 283 (1954).
(266) C. E. Jensen, *Acta Chem. Scand.*, **7**, 603 (1953).

hyaluronic acid with a relative viscosity, at a concentration of 1 g./l., of 68.5 and 76.4 (compare Ref. 107). Other samples had values of 11 to 12. The molecular weights of the potassium salts of the samples, determined osmotically,[267] were 554,000 and 1,750,000 for the "high" samples and 517,000 to 527,000 for those possessing a lower relative viscosity. The importance of isolation techniques which avoided degradation was stressed. Values of η_{sp} were a linear function of the molecular weights,[268] from which relation a flexible, linear molecule was suggested. An electron-microscopic study of the potassium salt indicated[269] a length of several thousand Å. and a width of less than 30 Å., whereas sedimentation and diffusion measurements[270] gave a value of 520,000 for the molecular weight. (For $c = 0.1\%$, $S_{20} = 3.0 \times 10^{-13}$; $D_{20} = 1.0 \times 10^{-7}$; $\bar{V} = 0.860$; $f/f_0 = 3.8$.) A molecular weight of 270,000, determined osmotically, for potassium hyaluronate from the vitreous body of cattle has also been reported.[271]

The difficulties of isolating hyaluronic acid without occurrence of degradation have also been stressed by Ogsten and Stanier.[272] These authors suggest that a method involving ultrafiltration is essential. The 27 % of protein in the product was thought to be an integral part of the molecular complex *in vivo*. A study of sedimentation, viscosity, and streaming birefringence indicated, in contrast to Jensen's results, that the native hyaluronic acid complex is a random coil of nearly spherical shape and is very highly hydrated. A molecular weight of about 1.2×10^7 was indicated by these results.

Light-scattering measurements have indicated a higher molecular weight for the hyaluronic acid isolated from umbilical cord.[273] In solutions of *p*H 1.8 to 7.0 and of ionic strength 0.12, values of $(2.8 \text{ to } 3.4) \times 10^6$ were obtained. The data were interpreted as indicating a somewhat rigid coil of radius 2200 Å. However, at lower ionic strength (0.012), a higher molecular weight [$(4.8 \text{ to } 6.5) \times 10^6$] was found. The cause of this effect is not known.

The labile nature of the polysaccharide and its complex obviously makes study of this material difficult.

(267) J. A. Christiansen and C. E. Jensen, *Acta Chem. Scand.*, **7**, 1247 (1953).
(268) C. E. Jensen, *Acta Chem. Scand.*, **8**, 937 (1954).
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(271) J. A. Christiansen and C. E. Jensen, *Acta Chem. Scand.*, **9**, 1405 (1955).
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The Alkali-stability and Molecular Size of Glycogens

By C. T. Greenwood and D. J. Manners

(Department of Chemistry, University of Edinburgh)

In view of present interest in the alkaline degradation of polysaccharides, and in particular, of starch[1] and its component amylose[2] and amylopectin,[3] we now report the effect of alkali on the molecular size of glycogen.

The classical Pflüger method for the preparation of glycogen involves digestion of the tissues with 20—60% aqueous potassium hydroxide at 100°, followed by precipitation of the glycogen with alcohol. The method has been criticised by Meyer and Jeanloz[4] who suggested that degradation of the glycogen occurred during the alkaline extraction. However, Bridgman[5] reported that glycogen extracted by the Pflüger method, or with trichloroacetic acid from two halves of a rabbit liver had a similar molecular weight.

We have determined the sedimentation constants of four samples of glycogen isolated from the halves of two rabbit livers, as shown in the Table:

	Sample	Method of extraction	Sedimentation const., $\times 10^{13}$ (c.g.s. units)
Liver A	1	Hot water	85
	2	Pflüger	86
Liver B	3	Hot water	76
	4	Pflüger	83

It is concluded that, in the presence of air, the extent of degradation of glycogen by 30% aqueous potassium hydroxide at 100° is no greater than that which might be caused by the action of boiling water. These results are in agreement with those of Staudinger[6] who showed that the molecular weights of guinea-pig liver and muscle glycogens were unaffected by digestion with 15—30% potassium hydroxide at 100° for 1 hour.

By contrast, hot dilute alkali appears to degrade glycogen. Digestion of another sample of rabbit-liver glycogen in 8% sodium hydroxide solution at 100° for 1·5 hours caused a reduction in the sedimentation constant from 86 to 57 $\times 10^{-13}$ c.g.s. units and an increase in polymolecularity[7] of the polysaccharide, as shown by a broadening of the peak of the schlieren pattern.

In continuation of physicochemical studies on starch-type polysaccharides, the sedimentation constants of 17 other samples of glycogen have been determined. The majority of the samples, from vertebrate and invertebrate tissues, were isolated by the Pflüger method, and the sedimentation constants were found to vary between 39 and 130 $\times 10^{-13}$ c.g.s. units. A mean value of 1·5 $\times 10^{-7}$ being assumed for the diffusion constant of glycogen,[8] these results correspond to molecular weights of 2—6 $\times 10^{6}$. All the samples were polymolecular. In addition, six of the glycogens were *polydisperse* and showed the presence of a second component, in some instances heavier and in others lighter than the bulk. These glycogens are unusual, although the polydispersity of samples of human-liver glycogen has previously been reported.[9]

It has been suggested[10] that purification of glycogen by precipitation with glacial acetic acid may render it unsuitable for ultracentrifugal analysis. The sedimentation constant and schlieren pattern of rabbit-liver glycogen (sample 4) is, however, unaltered after four precipitations of the glycogen with 80% acetic acid.

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[*Received, November 7th*, 1956.]

[1] Baum and Gilbert, *Chem. and Ind.*, 1954, 489.
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[5] Bridgman, *J. Amer. Chem. Soc.*, 1942, **64**, 2349.
[6] Staudinger, *Makromol. Chem.*, 1948, **2**, 88.
[7] The term "polymolecular" is used to describe a chemically homogeneous polymer having a variation in molecular weight, whilst "polydisperse" denotes a polymer system containing more than one component.
[8] Bell, Gutfreund, Cecil, and Ogston, *Biochem. J.*, 1948, **42**, 405.
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*The Sedimentation Behavior of the Components of Potato Starch in Dilute Alkali**

Very few sedimentation measurements have been carried out on adequately purified and characterized starch components, notwithstanding the valuable information that this method can give regarding molecular weight and its distribution.[1] Studies on the unsubstituted components in aqueous solution are complicated by the tendency for both the linear amylose- and the branched amylopectin-components to aggregate. Previous experiments[2] have been carried out on derivatives dissolved in organic solvents, but we have found that sedimentation measurements may conveniently be made on the components dissolved in dilute alkali. Such solutions are stable for the length of time required for measurement, and the results are reproducible. This method therefore avoids complications due to degradative effects during the formation of derivatives.[1]

Samples of amylose and amylopectin isolated from potato starch have been investigated. Fractionation was carried out under conditions involving an oxygen-free atmosphere.[3] The amylose was precipitated first as the thymol complex, and then purified by repeated crystallization using butanol. The resultant complex was dehydrated with butanol and dried.[3] Amylopectin was obtained by freeze-drying directly the supernatant from the thymol-amylose complex. The two components were characterized by potentiometric measurements of iodine binding power under standard conditions.[4] The amylose bound 19.5% of iodine, and the amylopectin [illegible]% 0.09 % (corresponding to less than 0.5% of linear material). Both components were dissolved directly before use in 0.2 *M* potassium hydroxide on shaking at room temperature.

Sedimentation rates were determined using a Spinco ultracentrifuge and a cell incorporating a Kel-F centerpiece. Preliminary experiments showed the optimum speed for solutions of amylose of concentrations greater than about 0.1 g./100 ml. was 60,000 r.p.m., while for more dilute solutions, 30,000 r.p.m. was more suitable. Amylopectin solutions were spun at either 30,000 or 15,000 r.p.m., depending on the concentration. Careful observation of the Schlieren patterns during the acceleration period showed that there was no rapidly sedimenting material. Analysis of the Schlieren diagrams showed that over 90% of each polysaccharide was present in solution. The results for typical runs are shown in Figures 1 and 2, where an acid-degraded amylose and amylopectin[5] and a glycogen[6] are also included for comparison.

Both components show a concentration dependence of the sedimentation constant; in the case of the undegraded amylopectin it is extremely large. This result is consistent with the concept of a linear amylose molecule, but it is unexpected for the branched component which is usually thought to be more spherical.[1] These results may indicate, however, that amylopectin is an elongated molecule. With increase in concentration, the outer

* This is Part IV in the series "Physicochemical Studies on Starches."

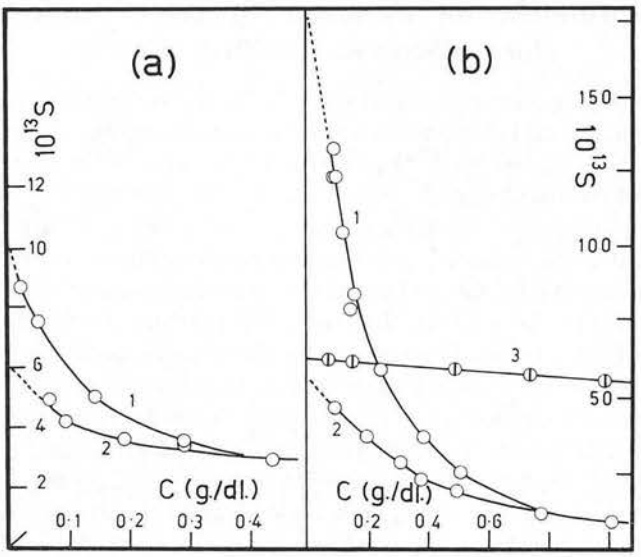

Fig. 1. The variation of sedimentation constant (S) with concentration (C) for the starch components in 0.2M-KOH. (a) Amylose (curve 1) and acid-degraded amylose (curve 2). (b) Amylopectin (curve 1) and acid-degraded amylopectin (curve 2). Curve 3 is yeast glycogen.

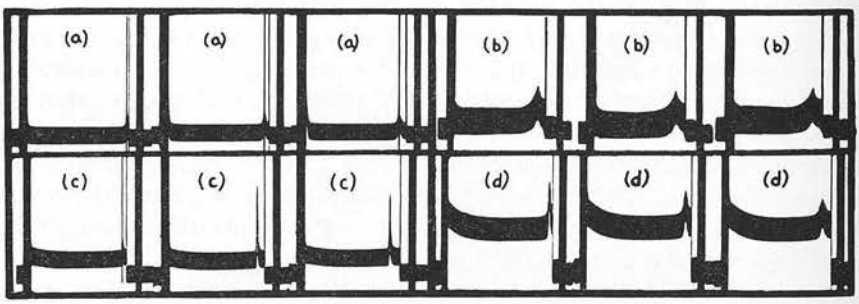

Fig. 2. Typical sedimentation diagrams for: (a) amylopectin: C = 0.79 g./dl.; speed = 30,000 r.p.m.; 5, 12, and 19 min., respectively, after reaching full speed; angle of Schlieren bar = 80°. (b) Amylopectin: C = 0.18 g./dl.; speed = 15,000 r.p.m.; 10, 12, and 14 min., respectively; angle of Schlieren bar = 40°. (c) Amylose: C = 0.30 g./dl.; speed = 60,000 r.p.m.; 10, 18, and 25 min., respectively; angle of Schlieren bar = 70°. (d) Amylose: C = 0.08 g./dl.; speed = 60,000 r.p.m.; 3, 7, and 12 min., respectively; angle of Schlieren bar = 45°.

chains of the highly branched amylopectin might well entangle, and strong secondary valency forces must occur even in the alkaline media. It is of interest that the sedimentation constant for glycogen is relatively independent of the concentration. This is further evidence of a fundamental difference in structure between the two branched glucans.[1]

For the starch components, free sedimentation only occurs at low concentrations (<0.2 g./100 ml. for amylose, and <0.4 g./100 ml. for amylopectin), and it is therefore necessary to carry out experiments at extremely

low dilutions. Unfortunately, lower limits of 0.02 g./100 ml. and 0.1 g./100 ml. for amylose and amylopectin, respectively, were fixed in these experiments by the sensitivity of the optical system. It is suggested that lower dilutions may be examined by the use of an alkali-resistant synthetic boundary cell. This is being investigated.

These experiments are being extended to include measurements of the appropriate diffusion constants so that estimates of molecular weight and shape can be obtained. Preliminary results indicate a value of the order of 5,000 for the DP of the amylose obtained by the above fractionation method.

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5. J. M. G. Cowie and C. T. Greenwood, unpublished experiments.
6. Supplied by Dr. D. J. Manners.

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*The Dose Rate Dependence of Kel-F Degradation by Ionizing Radiation**

In this study films of Kel-F 300 (polymonochlorotrifluoroethylene) were subjected to beta irradiation from strontium-90 sources of various intensities and the time for dielectric breakdown to occur was noted. The experimental arrangement is shown diagrammatically in Figure 1, and was used basically to measure the conductivities of three-mil thick Kel-F films while they were being irradiated in air.

Dielectric breakdown occurred when the films degraded physically into yellow powder. Table I shows the time T_D in days for degradation and short circuiting to occur as a function of beta source current density I_B in micromicroamp/cm.^{2}

* Based on a paper presented at the Winter General Meeting of the American Institute of Electrical Engineers, New York, N. Y., January 23, 1957.

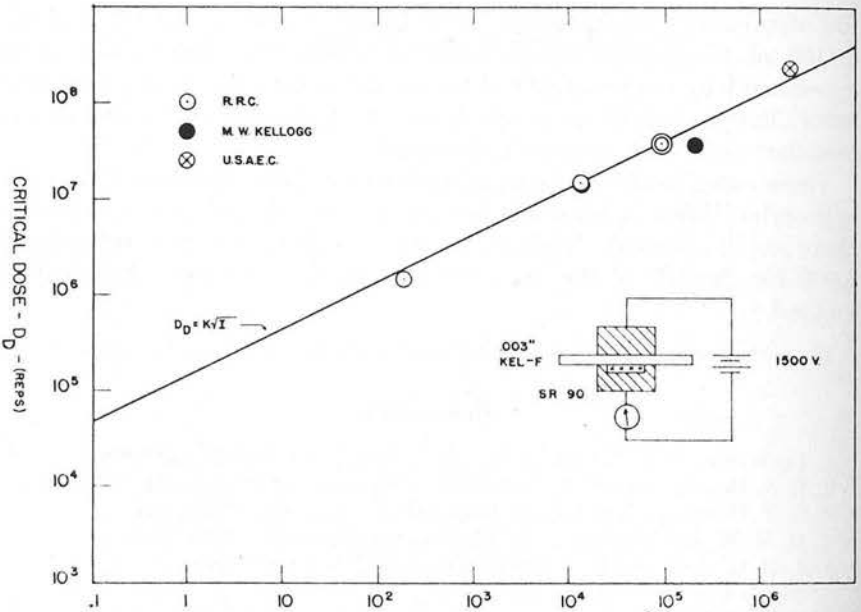

Fig. 1. Critical dose *vs.* radiation rate for Kel-F.

The current density I_B measured for each source is converted to reps/hr. by the relationship $^1/_4$ rep/sec. = 1 micromicroamp. The dose for degradation D_D was calculated by $D_D = IT_D$. These results are plotted in Figure 1 and closely fit the solid line representing the equation:

$$D_D = KI^{1/2}$$

where K is a constant. This result indicates the dose for degradation is not constant but depends on radiation intensity.

According to F. Honn[1] of the Minnesota Mining and Mfg. Corp., manufacturers of Kel-F, the degradation dose for gamma irradiation of Kel-F 500 is 4×10^7 reps at an intensity of 2×10^5 reps/hr. Sisman and Bopp[2] report that the tensile strength of Fluorothene, made by the Union Carbide Corp., drops to zero at a reactor dose of 2.5×10^{17} NVT of neutrons and gammas at a pile intensity of two megareps/hr.; using their conversion factor of 10^9 NVT/rep for the CH_2 group, one obtains $D_D = 250$ megareps.

TABLE I

	I_B, $\mu\mu$amp./cm.^{2}	I, reps/hr.	Temp., °C.	T_D, days	D_D, megareps
(1)	100	90,000	80	20	40
(2)			30	20	40
(3)	13	12,000	80	59	15
(4)			30	54	14
(5)	0.2	180	30	340	1.5

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MECHANISM OF THE DEGRADATION OF POTATO AMYLOSE BY β-AMYLASE

By J. M. G. Cowie, I. D. Fleming, C. T. Greenwood and D. J. Manners

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Although the general action pattern of β-amylase on amylose is well-established, the specific reaction mechanism is uncertain, and has been the subject of much controversy.

Swanson,[1] on the basis of iodine absorption measurements, and Kerr and Cleveland,[2] using physical measurements, suggested that the mechanism is of the " single-chain " type in which the enzyme attaches itself to one amylose molecule and degrades it completely before attacking another. However, Bourne and Whelan[3] and Hopkins and Jelinek[4] have interpreted their iodine absorption measurements as indicating a " multi-chain " mechanism whereby all the amylose molecules are degraded simultaneously. More recently, Hopkins and his collaborators[5] have also suggested that β-amylase degrades short-chain amylose molecules by multi-chain action.

If the mechanism is of the single-chain type, then under normal experimental conditions, with a large substrate/enzyme ratio, the degree of polymerization ($\overline{D.P.}$) of the residual amylose at any time during the reaction will be the same as that for the original up to the stage when the number of substrate molecules is approximately equal to the number of enzyme molecules. (At this latter point, a multi-chain mechanism is then inevitable.) On the other hand, if a multi-chain mechanism is operative throughout, then the $\overline{D.P.}$ of the amylose will decrease as the reaction proceeds, the reduction being proportional to the percentage conversion to maltose. The reaction mechanism can therefore be established unequivocably from measurements of the molecular weight of the residual amylose at varying stages of β-amylolysis.

Experiments of this type have been carried out by Kerr and Cleveland,[2] who isolated the polymer-product after 50% conversion to maltose and found the $\overline{D.P.}$, iodine affinity, and limiting viscosity number of this to be virtually the same as the original amylose. We have extended this treatment by measuring the $\overline{D.P.}$ of the residual polymer at varying degrees of conversion throughout the reaction, as well as the $\overline{D.P.}$ of a 50% conversion dextrin and a 75% β-limit dextrin.[6]

Amylose (prepared under oxygen-free conditions from potato starch as previously described[7] and having an initial $\overline{D.P.}$ of about 3500) was incubated at pH 4·6 and 35°C. with soya bean and barley β-amylases, which hydrolysed the polysaccharide to give 75% and 100% conversion to maltose, respectively. (The barley enzyme preparation showed Z-enzyme activity.[6]) Portions of each digest were examined at intervals, in 0·2M potassium hydroxide, in an electrically-driven Spinco ultracentrifuge; each fraction was examined at several dilutions in view of the concentration dependence of the sedimentation constant of amylose in alkali.[8]

The sedimentation constant of the original amylose was also determined and comparison of the results indicated that up to 75% conversion to maltose, the residual polymer product in both digests had, within the limits of experimental error, the same sedimentation constant as the original polysaccharide.

Furthermore, a detailed study of the sedimentation constants, limiting viscosity numbers,[7] and iodine affinities[7] of both a 50% conversion product (prepared by using barley β-amylase) and a 75% β-limit dextrin (obtained using soya bean β-amylase) showed that the properties of these polysaccharides differed only slightly from those of the original amylose.

On the basis of these results, it would appear that, in agreement with Kerr and Cleveland,[2] the mechanism of the action of the two β-amylases used here on pure potato amylose of high $\overline{D.P.}$ is of the " single-chain " type.

It is hoped to publish full details of these results and experimental methods elsewhere.

The authors wish to thank Prof. E. L. Hirst, F.R.S., for his interest in this work and the Rockefeller Foundation for financial assistance.

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LETTERS TO THE EDITORS

Electrophoresis of Polyphenylene

Solutions of polyphenylene in pyridine-water mixtures show electrical conduction far in excess of that of corresponding pyridine-water mixtures.[1] This observation obviously invites an electrophoretic investigation of the polymer.

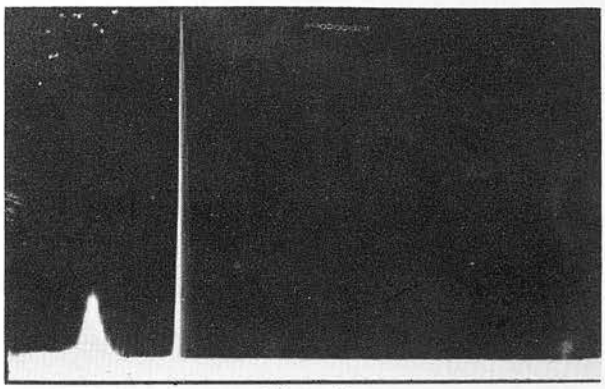

Figure 1.

Consequently, a solution containing 0.08 g./100 ml. of polyphenylene of a degree of polymerization of approximately 34 in a solvent of 1:20 pyridine-water (pH 8.8) was observed in a Spinco electrophoresis instrument at electric field strengths of 190 or 380 v./cm. In either case, one very sharp peak was observed (Fig. 1) whose rate of migration gives a mobility of 0.3 $\times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$. Excluding most unlikely coincidences, the results indicate an amazing uniformity of degree of polymerization. This can be explained by assuming a continuous decrease in the reactivity of the terminal chlorine with increasing chain length.

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The Degradation of High Polymers

During recent investigations of the degradation of the starch components, we have re-assessed methods of expressing the rate of polymer degradation.

Degradation studies are of great importance as they can indicate the presence of anomalous or "weak" linkages. In many instances throughout the literature, viscosity measurements ($[\eta]$ or η_{sp}/c) have been used to characterize the polymer degradation products, the rate of degradation then being expressed simply as the change of viscosity with time. The resultant curve (see, *e.g.*, Fig. 1a) has been interpreted as indicating the preferential scission of weaker bonds followed by a decreased rate of degradation to some limiting value. We wish to emphasize here that (*1*) changes in viscosity are *not* themselves directly a true measure of the degradation rate, and (*2*) the apparent limit in $[\eta]$ (or η_{sp}/c) is fallacious.

Considering first *qualitative* aspects of degradation, this process should be followed by number-average methods. Such methods are obviously insensitive to small initial changes, while weight-average methods (*e.g.*, viscometry) provide a sensitive measure of these. However, at high degree of degradation, when only relatively low molecular weight material is present, weight-average methods become insensitive to further changes and the measured quantity (say, viscosity) will then approach an apparent limit.

The correct formulation for measuring degradation can be obtained following the method of Ekenstam[1] and Schulz and Husemann:[2]

Consider a polymer system containing n gram moles of equally strong and accessible cleavable bonds/liter, then for a zero-order degradation reaction

$$dn/dt = -k_0$$

and for a first-order degradation reaction

$$dn/dt = -k_1 n$$

If there are w grams of polymer composed of repeating units of molecular weight (M_0), and having a number-average degree of polymerization (P), then the over-all condition must hold that

$$dP/dt = (dP/dn)(dn/dt)$$

But

$$n = w(P - 1)/PM_0$$

Hence:

$$dP/dn = M_0P^2/w$$

and for a *zero-order reaction:*

$$k_0 = w[P_t^{-1} - P_0^{-1}]/M_0t$$

where P_t and P_0 are the values for the number-average degree of polymerization at times t and 0, respectively.

Similarly, for a *first-order reaction*

$$k_1 = (1/t) \ln [(1 - P_0^{-1})/(1 - P_t^{-1})]$$

or

$$k_1 = (1/t)[P_t^{-1} - P_0^{-1}]$$

on expansion of the logarithm and ignoring terms higher than the first.

Hence for either a zero- or first-order reaction, the degradation rate constant in the initial stages is proportional *not to P but to* P^{-1}, and P^{-1} versus t will be linear. For large amounts of degradation, this relationship will still hold for a zero-order reaction, but ln $(1 - P^{-1})$ versus t is necessary for a first-order reaction as higher terms in the expansion are then required.

Thus if degradation is followed viscometrically and $[\eta] = KP$, the degradation rate constant is obtained *from the graph of* $[\eta]^{-1}$ *versus* t, and not $[\eta]$ versus t, in agreement with McBurney.[3] The presence of some rapidly degraded weak linkages will then be shown by $\lim_{t \to 0} [\eta]^{-1} \neq$ that for the original polymer. (If $[\eta] = KP^{\alpha}$, then degradation should be expressed as $[\eta]^{-1/\alpha}$ versus t.)

We have applied this concept to some results described in the literature for polystyrene and cellulose degraded by different methods. Results have been re-plotted as $\Delta[P^{-1}]$ versus t, rather than P^{-1} versus t to reduce the graphs to a common origin.

(a) **Polystyrene.** Figure 1(a) shows the $[\eta]$ versus t curves for the pyrolysis studies of Wall and co-workers[4] (curve 1) and Jellinek[5] (curve 2), and the ultrasonic studies of Melville and Murray[6] (curve 3). The corre-

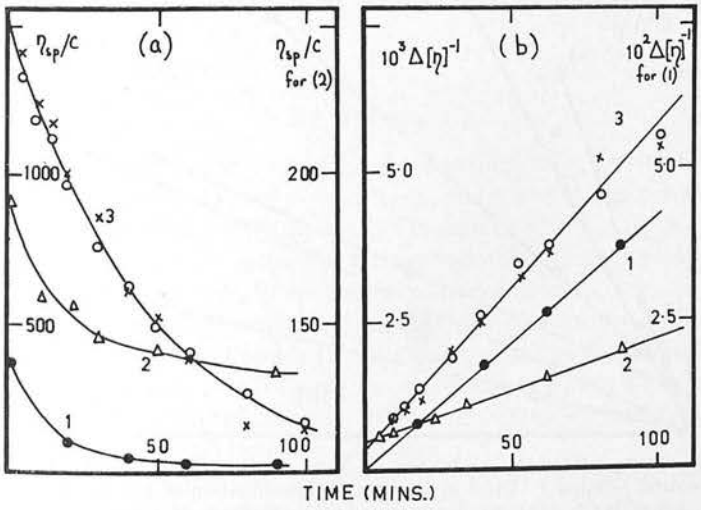

Fig. 1. (a) η_{sp}/c *vs* t for the degradation of polystyrene. Curve *1*. Pyrolysis of low molecular weight polymer.[4] Curve *2*. Pyrolysis of high molecular weight polymer.[5] Curve *3*. Ultrasonic degradation.[6] (b) $\Delta[\eta]^{-1}$ *vs.* t for the degradation of polystyrene. Curves *1–3* as for (a).

sponding plots of $\Delta[\eta]^{-1}$ versus t are shown in Figure 1(b). In all cases, there is *no evidence for a decrease in the degradation rate constant* with time. The results of Wall and co-workers[4] (who have already plotted their data as $[P_t^{-1} - P_0^{-1}]$ versus t) are included as an example of a system in which there is no evidence for either weak linkages or a decrease in degradation rate constant. However, for the higher molecular weight products studied by thermal methods,[5] and the lower molecular weight products studied by ultrasonic methods,[6] weak bonds are indicated (curves *2* and *3*, Fig. 1 (b)). This is in agreement with Jellinek's conclusions.[5] Such effects were not considered by Melville and Murray.[6] The results show further that a limiting value for molecular weight in ultrasonic degradation processes[6] is unlikely.

(b) Cellulose. Schulz and Husemann[2] have interpreted their results for the oxidative degradation of cotton cellulose as indicating the presence of weak links in the molecule. In Figure 2 some of these authors' data have been re-plotted (curves *1–4*). Degradation would appear to follow a zero-order reaction, as the rate constant varies approximately inversely to the concentration. Again, the rate constant does not alter with time. On the basis of the above, there is no evidence for weak bonds for the low concentrations (curves *1* and *2*), but indications of them for the higher concentrations (curves *3* and *4*). (It is of interest that Sharples[7] has suggested, from results plotted in a similar manner, that weak links are only introduced by the pretreatment of the cellulose.)

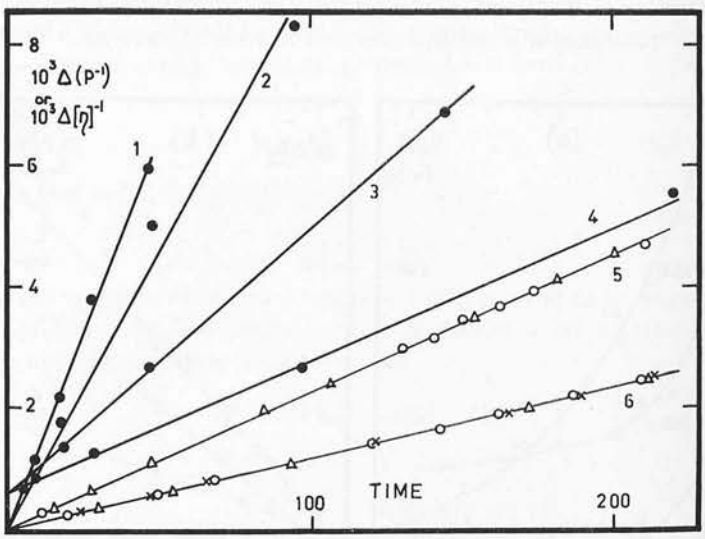

Fig. 2. Degradation rate constants for cellulose. Curves *1–4*. Oxidative degradation of cotton cellulose.[2] Time in minutes. Concentration of cellulose: 1, 2, 3, and 4 g./1., respectively. Curves *5* and *6*. Acid hydrolysis of methylated celluloses.[8] Time in hours. (Time-axis for curve 5 is doubled, *i.e.* curves *5* and *6* are actually coincident.) (○) Unfractionated commercial cellulose: curve *5*, concentration = 1.878 g./1.; curve *6*, concentration = 2.383 g./1. (×) Commercial cellulose fraction: concentration = 4.181 g./1. (△) Viscose rayon fraction: concentration = 2.488 g./1.

The results of Gibbons[8] for the homogeneous acid hydrolysis of various methylated celluloses are shown in curves *5* and *6* (Fig. 2). There is no concentration dependence for the rate constant. However, in contradiction to this author's conclusions, there appears to be conclusive evidence from the graphs that there are no weak bonds present in the methylated samples.

The authors wish to thank Dr. D. Taylor for helpful discussion.

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Applicability of the Stokes' Equation to Macromolecules

The Stokes' equation is

$$f = n\pi r\eta_0 v \tag{1}$$

where f is the frictional force on a sphere of radius r moving with a velocity v in a fluid continuum having a viscosity η_0, and where the numerical factor $n = 6$ has been verified experimentally for spheres of radius down to about 1300 A.[1] However, for molecules having radii of 3–6 A. (based on their van der Waals volumes V_w[2]) the numerical factor must be reduced to 5.[3, 4] It has been speculated previously[4] that for macromolecules such as proteins, which have radii of about 17 A. or more, equation (1) should be used with $n = 5$. However, it is shown below, using essentially the arguments of Polson[5] in another connection, that more probably the unmodified Stokes' equation is valid for these particles.

The effective hydrodynamic volume of a molecule is given by V_wH, where H is a "hydration factor" which equals unity when the molecule is unhydrated. It has been shown[6] that this volume may be used in applying the Einstein viscosity equation to solutions of small molecules, in the form

$$[\eta] = \frac{\nu N V_w H}{100M} \tag{2}$$

where $[\eta]$ is the intrinsic viscosity of the solution, ν the "viscosity increment" of the solute particle, N Avogadro's number, and M the molecular weight of the solute. On the other hand, the diffusion coefficient D of the solute is given by

$$D = \frac{kT}{n\pi\eta_0}\left(\frac{f_0}{f}\right)\left(\frac{4\pi}{3V_w H}\right)^{1/3} \tag{3}$$

where k is Boltzmann's constant, T the temperature, and (f/f_0) the "frictional ratio"[7] to correct, where necessary, for the nonspherical shape of the molecule. Then

$$V_w H = \frac{100[\eta]M}{\nu N} = \frac{4\pi}{3}\left(\frac{kT}{n\pi\eta_0 D}\right)^3\left(\frac{f_0}{f}\right)^3$$

or

$$D^3[\eta]M = \frac{4\pi\nu N}{300}\left(\frac{kT}{n\pi\eta_0}\right)^3\left(\frac{f_0}{f}\right)^3 \tag{4}$$

For a given molecule both ν and (f/f_0) are determined by its asymmetry, and for the special case of an ellipsoidal molecule they may be calculated from its axial ratio.[7, 8] Hence $\nu(f_0/f)^3$, which we shall designate as the "shape factor" s, may be calculated from axial ratios. Some values are given below.

Axial ratio	s (prolate ellipsoid)	s (oblate ellipsoid)
1.0	2.50	2.50
2.0	2.53	2.52
4.0	2.81	2.55
6.0	3.11	2.58
10.0	3.68	2.59

For solutions in water at 20°, equation (4) becomes

$$D^3[\eta]M = 5.33 \times 10^{-14} s/n^3 \tag{4a}$$

and so for $n = 5$

$$D^3[\eta]M \geqslant 10.6 \times 10^{-16} \tag{4b}$$

and for $n = 6$

$$D^3[\eta]M \geqslant 6.16 \times 10^{-16} \tag{4c}$$

Polson[5] found for aqueous solutions of the small molecules, pentaerythritol, DL-valine, α-alanine, and β-alanine, values of $D^3[\eta]M$ between $10.55\text{–}12.0 \times 10^{-16}$, in agreement with (4b). However, for aqueous solutions of glycine, sucrose, and glucose, $D^3[\eta]M$ was between $7.5\text{–}8.2 \times 10^{-16}$,

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516. *Physicochemical Studies on Starches. Part V.* The Effect of Acid on Potato Starch Granules.*

By J. M. G. Cowie and C. T. Greenwood.

The degradative effect of 0·2M-hydrochloric acid at 45° on the granular structure and molecular size of the components of two varieties of potato starch has been examined. The fractionated starch components were characterised by iodine-binding power, osmotic pressure, viscosity, and sedimentation. The mode of acid attack was similar for both samples. Under the conditions used, the granular structure was apparently unchanged, but both the amylose and the amylopectin component were degraded. The rates of degradation indicated that the amylopectin underwent preferential hydrolysis. The implications of these results with regard to granular structure are discussed.

Notwithstanding the large amount of work carried out on starch, little is yet known of the fine structure of the granule and the manner in which amylose and amylopectin are incorporated into it. It has been suggested that amylose is found in the granule preferentially at the centre, but it has also been maintained that it is evenly distributed throughout.[1] One approach to this problem is to study the effect of acid-treatment on the granule by estimating, after fractionation, the molecular size of the components. If the granule has an outer layer of amylopectin, the latter should be preferentially hydrolysed, whereas if there is uniform distribution both components should be degraded. In studies of the granular structure of potato starch, we have therefore examined such effects. Previous investigations along these lines have had contradictory results: Meyer and Menzi [2] reported that acid-treatment of potato and maize starch gives simultaneous degradation of both components, whilst Kerr [3] reported preferential degradation of amylopectin in maize starch. However, Meyer and Menzi [2] measured molecular weights by the dinitrosalicylic acid method, which has been shown to be unreliable for absolute molecular weights,[1] and Kerr [3] evaluated his osmotic data for amylopectin solutions by a method which has not yet been substantiated.[1] The studies reported here were undertaken in an effort to clarify the position, as all physical properties of the granule depend ultimately on granular structure and any method of tackling the latter problem is thus important.

Experimental Methods

Isolation and Purification of Potato Starch.—Potatoes (var. Redskin and Arran Banner severally) were thickly peeled, sliced, and then minced into ethanol to inhibit enzymic activity. After extraction with ethanol for 2 min. in an " Atomix " blendor, the pulp was filtered through muslin, and the filtrate *immediately* centrifuged. The supernatant liquid was discarded, and the starch washed by repeated sedimentation in 0·1M-sodium chloride. The residual pulp was then re-extracted 3—4 times with saline in the blendor. (Although extraction was obviously incomplete, the residual pulp was then discarded.) Starch rapidly sedimented from the filtrates. The starch-products were combined, suspended in 0·1M-sodium chloride, and shaken with toluene ($\frac{1}{10}$ vol.) overnight to denature protein.[4] After the granules had been allowed to sediment, the coagulated protein–toluene layer was discarded, and the extraction repeated four times to yield pure starch, which was stored in 0·1M-sodium chloride under toluene

* Part IV, *J. Polymer Sci.*, 1957, in the press.

at 0° (Arran Banner) or washed free from salt and stored under methanol at 0° (Redskin) (Found, for both samples: N, 0·02%).

Characterisation of Starch Products.—The efficiency of fractionation and the overall effect of acid-treatment on the granules was followed by measuring the amount of iodine bound by the various starch products. In view of the insensitivity of optical-absorption methods, the binding power was measured in the semimicro differential-titration apparatus previously described.[5] Titration conditions were: [iodide], 0·01M; pH, 5·85; temp., 20°.

The total free iodine in the starch solution was plotted against the mg. of iodine bound per 100 mg. of starch, and estimations of iodine affinity (the preferential uptake of iodine by the linear amylose component) were made by extrapolating the linear portion of the titration curves to zero free-iodine concentration. Calculations of the approximate percentage of amylose were made from the observed iodine affinity on the assumption that pure potato amylose bound 19·5% of iodine under these conditions.

Treatment of Granules with Acid.—Potato starch (10 g.; var. Redskin, washed free from salt) was stirred slowly (20 revs./min.) in 0·2M-hydrochloric acid (500 ml.) at 45° under oxygen-free nitrogen. Portions (125 ml.) were removed after 1, 2, 3, and 4 hr. severally (Expt. I). The starch was immediately washed 8—12 times with distilled water in the centrifuge until free from acid and then shaken with methanol for 24 hr. The samples were stored under methanol.

The above was repeated (Expt. II), except that starch samples (var. Arran Banner) were removed at intervals of ½, 1, 2, 3, 4, and 24 hr.

Estimation of Amount of Granule Solubilised by Acid.—A 2% suspension of starch in 0·2M-hydrochloric acid was kept at 45° for 1 hr. under nitrogen. A portion of the suspension was removed, centrifuged, and reduced in volume, and the amount of glucose obtained on hydrolysis by 3N-sulphuric acid for 2 hr. at 100° was estimated by the alkaline ferricyanide–ceric sulphate method of Lampitt, Fuller, and Coton.[6] The starch was re-treated with acid for 3 hr., and the additional glucose found in the supernatant liquors estimated. The residual starch granules were weighed.

To investigate whether oligosaccharides were present inside the granule after acid-treatment, starch (2 g.; treated with 0·2M-hydrochloric acid for 4 hr.) was gelatinised at 70° in water (400 ml.) for 1 hr. under nitrogen, and amylose precipitated with butan-1-ol. The supernatant liquors were then reduced to dryness, extracted with 50% methanol, and examined chromatographically (solvent: butan-1-ol–benzene–pyridine–water: 5 : 1 : 3 : 3; top layer; development time, 72 hr. at 18°).

Fractionation of Acid-treated Starches.—A slurry of starch in methanol was carefully added to vigorously stirred boiling water (500 ml.) under nitrogen, and boiling continued for 1½ hr. (Expt. I). (In Expt. II, the boiling was continued for 1 hr.) The solution was then allowed to cool to 60°, powdered thymol (0·5 g.) added, and the mixture set aside at room temperature (18°) for 3 days before the amylose–thymol complex was removed on the Sharples super-centrifuge. The amyloses were then purified by recrystallisation twice (Expt. I) from hot butan-1-ol saturated water, and were stored as the butan-1-ol complexes. Only one recrystallisation was carried out in Expt. II. Solid amyloses were isolated by stirring the butanol complex several times with butan-1-ol and then drying it *in vacuo* at 75°.

The amylopectin-containing supernatant liquors from the thymol–precipitates were freeze-dried directly, refluxed with methanol to remove thymol (3 times; 1½ hr. each), redispersed in water, and freeze-dried. In Expt. II, the supernatant liquors were extracted twice with ether before freeze-drying. This procedure yielded more soluble products.

Fractionation of Original Starch.—A 0·5% solution of starch was dispersed as above (1½ hr. for Expt. I and 1 hr. for Expt. II). After formation of the thymol complex, the amylose was reprecipitated three times with butan-1-ol and stored as this complex. Amylopectin was obtained as above.

Acetylation of Amyloses.—A portion of the butanol complexes of each of the amyloses from Expt. I was acetylated under conditions of minimum degradation[4] with pyridine and acetic anhydride at room temperature.

Estimation of Length of Unit Chain for Amylopectins.—The amylopectins from Expt. II were oxidised with sodium metaperiodate at 2° by Potter and Hassid's method[7] with the modification[8] that the liberated formic acid was estimated by titration to pH 6·25. The yield of formic acid was constant after 25 hr.

Measurements of Limiting Viscosity Number.—The specific viscosity ($\eta_{sp.}$) of the poly-

saccharide solutions was determined at several concentrations at 22·5°, and the limiting viscosity number $[\eta]$ determined graphically from the relation $[\eta] = \lim_{c \to 0}(\eta_{sp.}/c)$. Concentrations were expressed [9] as g./ml. Measurements were made on both of the unsubstituted components in M-potassium hydroxide, and on the acetylated amyloses in chloroform solution. Techniques were as previously described.[4]

Solution and concentration estimation was as follows :

(a) *For amyloses.* A portion of well-centrifuged butanol complex was dissolved directly in M-potassium hydroxide. Concentrations were then determined by hydrolysing a portion of the diluted resultant solution, and determining the reducing power as above.[6] The method was calibrated by standard solutions of glucose. Preliminary experiments indicated a recovery of 98—102% of glucose for hydrolysed " AnalaR " soluble starch. {Control experiments showed that the limiting viscosity number for a given sample was the same within experimental error whether solution had been achieved *via* the butanol complex or directly from the dried solid. However, the value of Huggins's constant (k') in the relation

$$\eta_{sp.}/c = [\eta] + k'[\eta]^2 c$$

varied.}

(b) *For amylopectins.* Solutions were prepared by weight.

(c) *For amylose acetates.* Concentrations were determined after measurements as before.[4]

Measurements of Osmotic Pressure (with W. N. Broatch).—Osmotic pressures (π) of the amylose acetates in chloroform solution were determined at 22·5° in the instrument previously described.[10] The membrane used was of No. 600 gel-Cellophane, which had been dehydrated by acetone and then conditioned to solvent. The cell constant, for a 0·04 cm. diameter capillary,[11] was independent of the meniscus level, and was reproducible within the setting of the cathetometer (±0·001 cm.). Any deviation from the cell constant which developed after use was thought to be due to contamination of the solution capillaries by evaporated polymer films (particularly with solutions of high viscosity) *although evaporation in this instrument is reduced.* The resultant polymer film is insoluble even after repeated washings of the solution chamber with solvent. Variations in cell constant were not therefore due to (*a*) solute adsorption on the membrane or (*b*) membrane dissymmetry effects. These " effects " observed previously [12] might be similarly explained. Although such variations of cell constant were avoided by working at a different level in the solution capillary, it was considered more satisfactory to dismantle and clean the apparatus.

Concentrations were estimated in duplicate after determination. The procedure adopted was to fill the osmometer with the most concentrated solution, determine the osmotic pressure, remove two volumes (by weight) for estimation of concentration, and add an equivalent volume of solvent. The solution was then carefully mixed, and the osmotic pressure of the diluted solution determined. Pressures were measured statically after an initial setting to 1·0 cm. below the expected value. There was no evidence of solute permeation of the membrane. In each experiment, the value of the intercept $(\pi/c)_0$ was obtained from the linear graph of π/c *versus* c, where π was expressed as dynes g.$^{-1}$ cm., and c as g. cm.$^{-3}$. Number average molecular weights ($\bar{M}_n$) were then calculated from van't Hoff's equation $\bar{M}_n = \boldsymbol{R}T(c/\pi)_0$.

Sedimentation Measurements.—Determinations were made with an electrically driven Spinco ultracentrifuge on both components dissolved in 0·2M-potassium hydroxide. Detailed results will be presented elsewhere.

Results

Examination of Acid-treated Whole Starches.—Microscopic examination showed that the granules which had been treated with acid for 4 hr. appeared unchanged and were still birefringent. However, the granules dispersed more readily to form a less viscous paste. The granules which had been subjected to 24 hr. treatment behaved similarly, although some split granules were visible.

Estimations of the amount of glucose in the acid supernatant liquors showed that after 1 hr. 0·2% and, after a further 3 hr., 0·8% of the granule was solubilised. Hence only about 1% of the total weight of the granules was solubilised as glucosans or reducing sugars in the 4 hr. treatment. Chromatographic examination of the gelatinised granules indicated that at the most only traces of oligosaccharides (probably tri- or tetra-glucosans) could have been present after acid-treatment.

An estimate of the apparent reducing power was obtained by titrating the starches directly

with the alkaline ferricyanide reagent. The results indicated that the reducing power increased regularly with time of acid-treatment as shown :

Time of acid-treatment (hr.)		0	1	2	3	4
No. ml. of M/100-ceric sulphate required/1 g. of starch	In N_2	0·40	2·80	5·00	7·40	9·83
	In O_2	0·10	4·90	8·75	13·92	18·40

An experiment in the presence of oxygen indicated an apparent greater increase in reducing power.

Potentiometric iodine-titration curves showed changes in iodine affinity of the samples: there was an apparent increase up to about 1 hr. followed by a decrease thereafter (Fig. 1*a* shows typical titration curves). In addition, the slope of the linear portion of the curve increased with

FIG. 1. (*a*) *Typical iodine-titration curves for acid-treated starches. The original potato starch is shown as the dotted curve. The numbers on each curve are the times of acid-treatment of the starch (in hr.).* (*b*) *Typical iodine-titration curves for acid-treated amylopectins. Potato amylopectin (containing 0·5% of amylose) is shown as the broken curve. Curves 1 and 2 are for amylopectins isolated after 3 and 4 hours' treatment, respectively.*

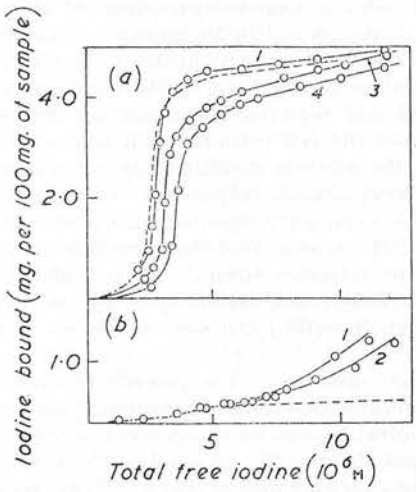
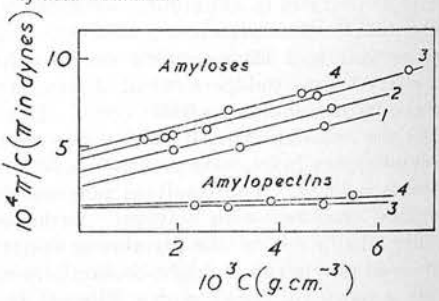


FIG. 2. *Graph of π/c* versus *c for the acetylated starch components in chloroform solution. The numbers on the curves represent the hours of acid-treatment.*



time of acid-treatment, as did the activity of free iodine necessary to saturate the linear amylose component.

Time of acid-treatment (hr.)		0	½	1	2	3	4
Redskin starch	Iodine affinity	4·03	—	4·25	—	3·89	3·10
	Amylose (%)	20·70	—	21·80	—	20·00	15·90
Arran Banner starch	Iodine affinity	4·03	4·51	4·30	4·11	3·60	3·07
	Amylose (%)	20·70	23·10	22·00	21·10	18·50	15·80

Properties of the Fractionated Components.—Potentiometric titration showed a normal curve (cf. ref. 4) for the amylose components, whilst the amylopectin curves were abnormal (Fig. 1*b*) and did not show the linear increase in bound iodine previously observed.[5] Fig. 2 shows the results of osmotic-pressure determination for the acetates from Expt. I. The properties of the separated fractions from both experiments are shown in Tables 1 and 2.

The results of physical measurements carried out on solutions of both components in alkali were reproducible, and the solutions were stable for the time required for measurement. The sedimentation measurements confirmed that depolymerisation effects, particularly in the case of the amylopectin components, were real.

Rates of Degradation.—Degradation of the components was expressed as (1) the number of bonds broken per initial polysaccharide molecule, and (2) the variation of $1/[\eta]$, both as a function of time. The use of the latter function instead of $[\eta]$ directly is justified as follows :

The number of bonds broken per w g. polysaccharide, $(q) = \frac{Nw}{M_0}\left(\frac{M_0}{M_t} - 1\right)$ where M_0, M_t

are the number-average molecular weights at times 0 and t, respectively and $\boldsymbol{N}$ is Avogadro's number. This can be rewritten as

$$q = \boldsymbol{N}w(1/M_t - 1/M_0)$$

or $$q \propto \frac{1}{M} \propto \frac{1}{[\eta]} \text{ since in this instance } M = K[\eta].$$

Hence the rate of change of $[\eta]$ with time is *not a direct measure of degradation*, in agreement with McBurney.[14]

TABLE 1. *Properties of the amylose components.*

Redskin amylose

Acid-treatment (hr.)	Iodine affinity	$[\eta]$ in M-KOH	Calc. $\overline{\text{D.P.}}$[a]	$[\eta]$ of acetate[b]	M. wt. from π	Obs. $\overline{\text{D.P.}}$[c]	$10^{13}S_0$[d]
0	19·5	470	3480	—	—	—	12·0
1	—	220	1630	370	470,000	1630	9·9
2	20·3	190	1370	335	400,000	1390	8·3
3	—	140	1040	305	302,000	1050	7·3
4	20·7	130	925	270	286,000	990	6·2

Arran Banner amylose

Acid-treatment (hr.)	Iodine affinity	$[\eta]$ in M-KOH	Calc. $\overline{\text{D.P.}}$[a]	$10^{13}S$[e]	Acid-treatment (hr.)	Iodine affinity	$[\eta]$ in M-KOH	Calc. $\overline{\text{D.P.}}$[a]	$10^{13}S$[e]
0	19·5	520	3850	13·0	3	—	177	1310	—
½	—	280	2070	—	4	—	152	1130	4·8
1	19·3	225	1670	7·1	24	—	73	540	2·9
2	19·8	201	1490	5·8					

[a] Calc.[13] from $\overline{\text{D.P.}} = 7{\cdot}4[\eta]$. [b] Measured in $CHCl_3$ solution. [c] Calc. from previous column. [d] Sedimentation constant in c.g.s. units at infinite dilution obtained by graphical extrapolation from $S = f(c)$. [e] Sedimentation constant in c.g.s. units at $c = 0{\cdot}075$ g./100 ml.

TABLE 2. *The properties of the amylopectin components.*

Redskin amylopectin.

Acid-treatment (hr.)	$[\eta]$ in M-KOH	$[\eta]$ of acetate[a]	M. wt. from π	Obs. $\overline{\text{D.P.}}$[b]	β-Amylolysis limit[c]	$10^{13}S_0$[d]
0	182	—	—	—	56·5	175
1	123	220	—	—	57·4	60
2	118	205	—	—	58·2	52
3	113	220	$1{\cdot}1 \times 10^6$	3800	58·1	43
4	105	200	$1{\cdot}0 \times 10^6$	3500	57·5	35

Arran Banner amylopectin.

Acid-treatment (hr.)	$[\eta]$ in M-KOH	Av. length of unit chain	$10^{13}S_0$[d]	Acid treatment (hr.)	$[\eta]$ in M-KOH	Av. length of unit chain	$10^{13}S_0$[d]
0	182	25	175	3	108	24	42
½	112	24	63	4	106	24	34
1	122	26	58	24	48	22	14
2	110	25	49				

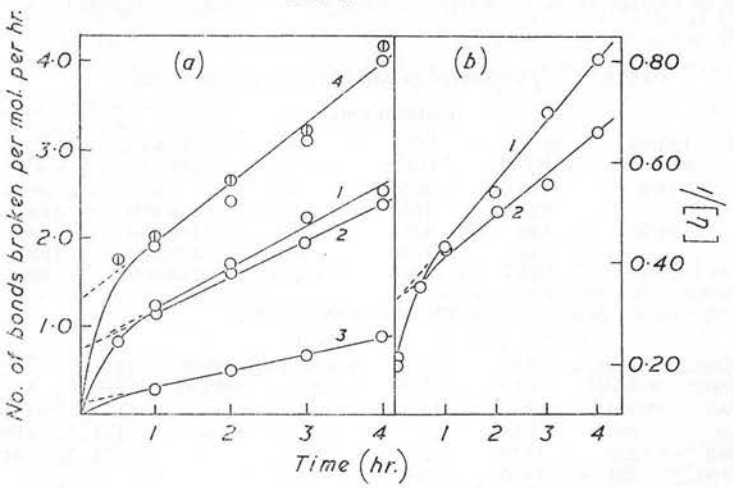
[a] Measured in $CHCl_3$ solution. [b] Calc. from previous column. [b] Results obtained through the courtesy of Mr. I. D. Fleming and Dr. D. J. Manners. [d] Sedimentation constant in c.g.s. units at infinite dilution obtained by graphical extrapolation from $S = f(c)$.

The graphs of (bonds broken) *versus* t, and of $[\eta]^{-1}$ *versus* t for the two series of amyloses are shown in Fig. 3. It is of interest that in neither instance do the linear portions of the curves extrapolate to the origin. In Fig. 3*a*, the linear portions of the curves correspond to rates of 0·5 and 0·4 (expressed as the number of bonds broken per initial amylose molecule per hr.) for the Redskin and Arran Banner amyloses, respectively. A linear relation (curve 3, Fig. 3*a*) was also found for $(S_0/S_t - 1)$ *versus* t, where S_0, S_t are the sedimentation constants at times 0 and t respectively. The rate of degradation from this curve (0·2) was less than the absolute rate because the diffusion constant for the linear amylose molecule must change considerably with decrease in $\overline{\text{D.P.}}$, and hence changes in S were not directly proportional to changes in $\overline{\text{D.P.}}$

For amylopectins, the degradation could not be calculated unambiguously in view of the

difficulty in obtaining absolute values for the molecular weight. However, again it was found that the graph of $(S_0/S_t - 1)$ *versus* t was linear, and on the assumption that for this component the diffusion constant was unlikely to vary considerably, the linear portion of this curve corresponded to a rate of 0·7 (units as above). This is likely to be a minimum rate as any disproportionate increase in the diffusion constant with time of degradation will increase the slope. Again the extrapolated linear portion of this curve did not pass through the origin.

FIG. 3.



(*a*) *Graph showing the number of bonds broken per initial polysaccharide molecule as a function of time.*
(1) *Redskin amylose.* (2) *Arran Banner amylose.* (3) $(S_0/S - 1)$ *for Redskin amylose.*
(4) $(S_0/S - 1)$ *for Redskin* (○) *and Arran Banner* (ⓘ) *amylopectins, respectively.*

(*b*) *Graph of* $1/[\eta]$ versus t *for the amylose components.*
(1) *Redskin amylose.* (2) *Arran Banner amylose.*

DISCUSSION

Experimental conditions were chosen such that our results are strictly comparable with those of both Meyer and Menzi [2] and of Kerr.[3] However, these authors presented insufficient data to permit comparison of actual *rates* of degradation of the two components. (Kerr's data unfortunately could not be replotted since " fluidities " were reported rather than times of acid-treatment.) At best, only general comparisons are possible.

The starches from the two varieties of potato (Redskin and Arran Banner) gave similar results, and hence can be considered to behave identically.

General Properties of Acid-modified Granules.—The action of acid does not alter the birefringent properties of the granule. There was no evidence of swelling in the acid media. It therefore appears that acid-treatment has preferentially affected the *amorphous* rather than the crystalline region of the granule. Sjoström [15] and Meyer and Bernfeld [16] observed similar effects, and Alsberg [17] also reported the treatment with hydrochloric acid (7·5%) destroyed the ability of the granule to swell and suggested that the portion responsible for swelling had been modified.

It was thought that estimation of the amount of reducing sugar produced in the acid supernatant liquor was the only suitable method for determining any loss in weight of the granule on acid-treatment. Only 1% was found after 4 hr. The possibility that oligosaccharides might have been produced and that these were unable to diffuse out readily from the persistent granular structure and so account for (1) the abnormal iodine titration curves for the whole starches and the amylopectins, and (2) the β-amylolysis results (particularly as the amylopectins were freeze-dried directly) was shown to be incorrect.

Potentiometric titrations indicated changes in the apparent percentage of linear material in the granule. These results can be explained by assuming that the material dissolving in the first hour is primarily amylopectin (the 0·2% of granular weight lost

accounting for a rise of 0·3—0·5% in the effective amylose-content), whilst that dissolving later is primarily amylose (the further 0·8% of granular weight lost being equivalent to about 4% of total amylose content).

Although Meyer and Menzi [2] reported no change in iodine-binding power of their acid-treated starches, it is of interest that the acid-modified maize starches studied by Schoch and his colleagues [18] also possessed iodine affinities very much lower than the original. The iodine affinity of starch can only be unaffected if the degradation products are solubilised completely (and there is no evidence for this), or acid-modified components have unaltered iodine-binding characteristics. However, although the fractionated amyloses possessed the usual iodine binding characteristics, all the amylopectin fractions bound more iodine than normal. The relative constancy of length of unit chain and β-amylolysis limits suggests that purely random hydrolytic action must have occurred, and no explanation can be advanced for this behaviour. The increased slope of the linear portion of the titration curves for the whole starches is probably accounted for on this basis.

The method of isolation of the amylose involved reprecipitations as the butan-1-ol complex which might involve the loss of some short chains. This was decreased in the case of Arran Banner samples by carrying out only one recrystallisation, although in neither experiment was there any apparent loss in amylose at this stage.

Degradation of the Components in the Granule.—The curves of rates of degradation (Fig. 3*a*) show that amylopectin is degraded considerably faster than amylose. Since the former must be a minimum rate, and also since the α-1 : 6-bonds (present to the extent of about 1 in 20) are reported [1] to be stronger than α-1 : 4-bonds, amylopectin must be preferentially degraded and is therefore far more accessible to attack than the amylose. The curves for both components do not extrapolate to zero time, which indicates (by comparison with cellulose chemistry) that acid-modification of the granule takes place in two stages, a rapid attack on amorphous regions, followed by a slower attack on more crystalline areas. This occurs for both components, but the results indicate a larger preferential scission of amylopectin rather than of amylose initially. (Although this two-stage process might well indicate the preferential scission of anomalous linkages, it is thought that in experiments involving whole starch granules such interpretations have to be made with caution.)

The results show that, even after 24 hr., degradation of amylose in the granule is relatively limited (to about 6 bonds per initial molecule). This contrasts markedly with the behaviour of the isolated amylose under similar conditions (preliminary unpublished experiments having indicated that such degradation is much more rapid), and suggests in contradiction to Lathe and Ruthven's conclusions [19] that penetration into the inner region of the *unswollen* granule is time-dependent and not instantaneous. Any *initial* attack will therefore occur preferentially in the surface layer of the granule before hydrogen ions diffuse into the inner regions.

The different rate curves for the two amylose fractions could be due to the prior storage of Redskin starch in methanol with subsequent dehydration and "splitting" of the granular structure permitting more ready access of reagent. This effect is not thought to be significant in these experiments.

Our degradation results appear to support Kerr's conclusions [3] that amylopectin is preferentially degraded. Ulmann [20] has reached a similar conclusion from chromatographic evidence. In view of the relatively small changes in the number of bonds broken, the changes in reducing power observed by Meyer and Menzi [2] can probably be explained by the "peeling action" [21] of their reagent.

Granular Structure.—Little is yet definitely known,[1] although crystallinity is evident in all starch granules (including those of the waxy variety). The formation of "crystallites" by the alignment of the long unbranched amylose molecules is very likely, but in potato starch granules amylose accounts for only 20% of the structure. The question

of the compacting of the remaining 80% is far more difficult, even to account for the reported *total* 50—60% crystallinity of the granule.[22] In particular, it is difficult to see how amylopectin molecules can pack themselves to any degree of crystallinity if they have the three-dimensional structure envisaged by Meyer. As suggested by Greenwood,[1] a two-dimensional structure might be more likely. Much depends on the actual shape of the amylopectin molecule, and experiments are in progress to determine this.

Unless amylose exists preferentially at the surface (no evidence exists for this), the surface layer of the granule must be predominantly amylopectin by virtue of its amount. (Evidence for the existence of a true amylopectin outer layer is contradictory, but unpublished experiments have indicated that such a layer may exist.) The amylopectin is most likely to form the amorphous regions of the granule, although, as Meyer has suggested, the outer branches of several amylopectin molecules might well align themselves to form a small crystallite. In addition, the outer branches might well link up with amylose in the crystalline regions, and amylose (particularly the short-chain material) might well form part of the amorphous region. Swelling of the granule would be associated with the amorphous branched component.

This concept of granular structure is consistent with the effects observed on acid-treatment. The primary attack of acid on the potato granule is on the surface membrane, because of the very limited swelling at the temperature of the experiment. The more freely accessible outer chains of the surface amylopectin molecules might well be degraded sufficiently to dissolve completely. This occurs mainly in the first hour, and is accompanied by a simultaneous attack on amylose and amylopectin in the amorphous regions. Degradation of the components arises by diffusion of the acid into the granule in the absence of swelling, and degradation takes place throughout the granule, but at a decreased rate in the crystalline regions. The hydrogen bonding responsible for the crystalline structure must be relatively strong by virtue of the limited hydrolytic effects observed even after 24 hours' treatment.

In view of the complex, but ordered, nature of the structure of the granule, it is not surprising that the mode of action of the acid is complex. Further methods of studying granular structure are being examined.

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559. *Physicochemical Studies on Starches. Part VI.* Aqueous Leaching and the Fractionation of Potato Starch.*

By J. M. G. Cowie and C. T. Greenwood.

A critical study has been made of the effect of aqueous leaching at various temperatures on potato starch granules. The efficiency of this procedure for fractionating starch has been followed by measuring the purity and molecular weight of the resultant components. Water leaches preferentially short-chain linear material, and its action is both inefficient and incomplete compared with the conventional methods involving complete disruption of the granular structure and formation of the amylose–thymol complex. The effect of aqueous leaching on granular structure is discussed. Potato amylose has a number-average degree of polymerization ($\overline{D.P.}$) of about 4000 glucose units. The relations, $\overline{D.P.} = 7{\cdot}4[\eta]$ for potato amylose fractions in M-potassium hydroxide, and $[\eta] = 4{\cdot}30(\overline{D.P.})^{0{\cdot}61}$ for the acetates in chloroform, are suggested.

Although the two components of starch can be relatively easily separated, little is known of how they are incorporated into the granule and how the physical nature of the granule governs the efficiency and ease of fractionation. Methods of fractionation have been recently reviewed; [1] the most satisfactory ones so far involve complete dissolution of the granule followed by the addition of a polar organic molecule to form an insoluble amylose complex. These procedures cannot give much information regarding granular structure, but some can be obtained by aqueous leaching. This method has often been used [2-4] to fractionate starch, although reports of its efficiency are at variance and there are insufficient accurate data regarding the purity of the fractionation products and their molecular weights. In continuance of studies of potato starch—its structure, methods of fractionation, and measurements of the size and shape of its components—we now report the results of a series of aqueous leaching experiments and estimations of the purity and molecular weight of the resultant fractions. Various features regarding the effect of leaching on granular structure are discussed, and its efficiency for fractionation is compared with other methods.

Experimental Methods

Preparation of Starches.—Starches were prepared from several varieties of potato by the method previously described.[5]

Fractionation Procedures.—(a) *Leaching at* 98°. Starch suspension (0·5% in 0·1% sodium chloride) was deaerated at room temperature (stream of oxygen-free nitrogen for 15 min.). The suspension was then placed on a vigorously boiling water-bath and stirred for 5—7 min. under nitrogen. The resultant gelatinized mixture was then cooled and centrifuged at 20,000 g. (preparative rotor of Spinco ultracentrifuge) for 10 min. Supernatant liquors were then removed, saturated with butan-1-ol, and kept at room temperature overnight. The sedimented granules were washed with distilled water (6 times), redispersed in saline, and heated for a further 5—7 min. The solid obtained after centrifugation was washed free from salt and freeze-dried directly, to give the " amylopectin " fraction. (Further experiments indicated

* Part V, *J.*, 1957,

that the gelatinized granules would not readily sediment in the absence of salt in the above force-field.)

(b) *Complete dispersion of granular structure.* Starch granules (0·5% in water) were stirred vigorously at 100° for ½—2 hr. under nitrogen. After cooling to 60°, thymol (1 g./l.) was added, and the mixture set aside for 3 days at room temperature. The amylose–thymol complex was then removed on the Sharples supercentrifuge and recrystallized three times from hot saturated butan-1-ol solution. The amylopectin-containing supernatant liquor (from the thymol complex) was freeze-dried, refluxed with methanol (3 times), dissolved in water, and freeze-dried again. (Later experiments showed that a more soluble product was obtained if treatment with methanol was avoided.[5]).

(c) *Combination of aqueous leaching at* 70° *and dispersion at* 100°. Starch suspension (0·5% in water) was stirred at 70° for 1 hr. under nitrogen. (Gelatinization occurred within 10 min.) The mixture was allowed to cool and centrifuged. After decantation of the supernatant liquor and filtration through a sintered-glass filter (G3), excess of butan-1-ol was added. Immediate amylose-complex formation was observed, but the mixture was kept for 24 hr. at room temperature for complete precipitation. The sediment of gelatinized granule residues was redispersed in water and boiled for 1½ hr. under nitrogen before fractionation as above.

Characterization of Fractionation Products.—(a) *Amylopectin.* The amount of amylose contaminating the branched component was estimated by potentiometric determinations of the amount of iodine bound.[6] The linear portion of the iodine sorption curve was extrapolated to zero free-iodine concentration; the ratio of the value of the intercept to that for pure potato amylose (19·5%) gave the amount of linear material present.

(b) *Amylose.* Purity was determined by titration as above. Molecular-weight determinations were made by measurements of (i) limiting viscosity number of the free component in M-potassium hydroxide at 22·5°, (ii) rates of sedimentation in 0·2M-potassium hydroxide, and (iii) osmotic pressure of the acetate in chloroform solution (at 22·5°). (For details of methods and procedures see ref. 5. The osmotic-pressure measurements were carried out in collaboration with Dr. W. N. Broatch.) Fig. 1 shows the experimental osmotic-pressure results.

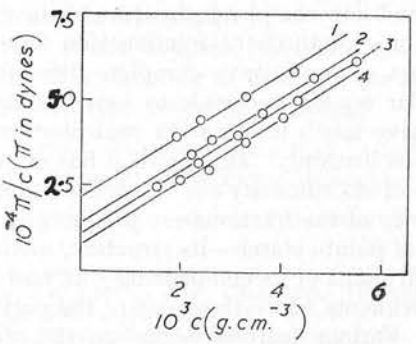


FIG. 1. *Graph of* π/c versus c *for the acetylated amyloses in chloroform solution.*

(1) *Amylose leached at* 70°. (2) *Amylose dispersed for* 1 *hr. at* 98°. (3) *Amylose dispersed for* 2 *hr. at* 98°. (4) *Amylose dispersed at* 98° *after leaching at* 70°.

RESULTS

The effect of aqueous leaching and fractionation was found to be identical for the two varieties of potato starch investigated, Redskin and Arran Banner.

Aqueous Leaching at 98°.—The results of typical experiments are shown in Table 1. Small-scale experiments indicated that successive leaching for two periods of about 7 min. yielded the purest amylopectin. The amyloses produced vary from 80% to 90% purity from the first leaching, but, on re-extraction, their purity drops to 60%.

Microscopic observation showed that at the end of 5 min. the granules were all swollen but mainly intact. The residual " sacs " stained mauve with iodine and blue-staining amylose had diffused into the supernatant liquors. Some granules still had accumulations of amylose towards one end, whilst others appeared to have been completely ruptured and to be devoid of any blue-staining material.

Whilst small-scale experiments proved relatively easy, leaching on a large scale (3 l.) was not successful. The fluid-like consistency of the gelatinized granules made their removal on the Sharples supercentrifuge extremely difficult and, further, on re-extraction, a higher proportion of amylopectin went into solution to yield a cruder amylose.

TABLE 1. *Properties of the products obtained by leaching at 98°.*

		Amylopectin		Amylose		
Expt.[a]	Leaching time (min.)	Purity (%) †	Amylose impurity (%) ‡	Initial purity (%)	$[\eta]$ in M-KOH [b]	$\overline{D.P.}$ calc.[c]
1—5 S	2 × 5—2 × 10	96—98	10—20	80—90	—	—
6 S	7	95	25	90	—	—
7 S	5	93	35	90	340 *	2520
8 S	2 × 5	96	20	90	365 **	2700
1 L	2 × 7·5	97	15	62	340 ***	2500 [d]

† Calc. from iodine uptake. ‡ Calc. from (iodine uptake ÷ 20) (see ref. 6.)
[a] S = small scale (50—100 ml.); L = large scale (3 l.). [b] Limiting viscosity number $[\eta]$ of recrystallized amylose in M-KOH at 22·5°. [c] Calc. from $\overline{D.P.} = 7{\cdot}4[\eta]$. [d] A value of 2500 was obtained from osmotic-pressure measurements on the acetate in chloroform solution; $[\eta]$ of acetate = 520.
* ** *** 1, 2, and 3 recrystallizations, respectively.

TABLE 2. *Properties of amylopectins and recrystallized amylose obtained from dispersions.*

	Amylopectin		Amylose			
Dispersion time (hr.)	Purity (%) [a]	Amylose impurity (%) [a]	$[\eta]$ in M-KOH [a]	$[\eta]$ of acetate [b]	$\overline{M_n}$ [c]	$\overline{D.P.}$ [d]
½	98·8	6	490	—	—	3600
1	99·2	4	520	680	1,125,000	3900
1½	—	—	470	—	—	3500
2	99·5	2·5	450	640	940,000	3260

[a] As for Table 1. [b] Measured in $CHCl_3$. [c] Determined from osmotic-pressure measurements on the acetate in $CHCl_3$ solution. [d] Calc. from previous column, or from $\overline{D.P.} = 7{\cdot}4\,[\eta]$.

TABLE 3. *Properties of components from combined leaching and dispersion.*

Expt.	Fraction	Purity [a]	Amylose impurity (%) [b]	$[\eta]$ in M-KOH	$[\eta]$ of acetate [b]	$\overline{M_n}$ [b]	$\overline{D.P.}$ [b]
70° leach	Amylose	94—98	—	240	480	527,000	1830
	Amylopectin	88	60	—	—	—	—
98° dispersion ...	Amylose *	100	—	560	770	1,534,000	5300
	Amylopectin	99·8	1	178	—	—	—

[a] Before recrystallization in case of the amylose. [b] As in footnotes to Table 2.
* Recrystallized twice.

Complete Dispersion of Granular Structure.—The results of these experiments are shown in Table 2. Thymol–amyloses were found to be about 75% pure, but recrystallization with butan-1-ol gave products binding 19·5% of iodine. Microscopic observation showed that after 30 minutes' dispersion, a few granules were still gelatinized, but not completely disrupted: after 1 hr. only occasional fragments of disrupted granules were apparent.

Leaching at 70° followed by Dispersion at 98°.—The results are shown in Table 3.

DISCUSSION

Aqueous Leaching at 98°.—The qualitative results of previous workers [2,4] were confirmed. Aqueous leaching of potato starch granules at 98° for a short time was shown to result in the extraction from the granule of material which is predominantly amylose, with the consequent formation of a residual granular network which is predominantly amylopectin. However, the extracted amylose (1) is contaminated with amylopectin (the latter being presumably of low molecular weight), and (2) possesses a much lower $\overline{D.P.}$ (2500) than that obtained from a conventional dispersive fractionation (cf. Tables 1 and 2). At the same time, 10—20% of the amylose is retained in the swollen granules.

These results suggest that preferential extraction of shorter amylose chains is occurring, owing to incomplete disruption of the granular structure and consequent inability of the larger amylose chains to diffuse out.

Aqueous leaching at 98° is therefore not suitable for the preparation from potato starch of either amylose of a high $\overline{D.P.}$, or amylopectin of high purity.

Dispersion Experiments.—Dispersion for 1 hr. resulted in amylose having a $\overline{D.P.}$ of 3900 glucose units, a value much larger than that from the above leaching experiments. After ½ hour's boiling, disintegration of the granule is incomplete with consequent retention of some material of high molecular weight (see below). Prolonged boiling results in hydrolysis, even under the oxygen-free conditions of the experiment. The amylopectins were considerably purer than those obtained by leaching.

Fractionation involving complete disruption of granular structure results therefore in the simultaneous production of amylose of high $\overline{D.P.}$ and pure amylopectin.

Leaching at 70° followed by Dispersion.—Leaching at 70° removed about 40% of the total amylose from the granule. The leached amylose-product was 97—98% pure (compare leaching at 98°), but was smaller ($\overline{D.P.}$ 1830). By comparison, the amylose subsequently isolated after dispersion of the residual granular structure had a very high $\overline{D.P.}$ of 5300 glucose units. The amylopectin obtained after this dispersion was the purest obtained in this work (see Table 3).

These results suggest, in agreement with Meyer and his co-workers [7] that subfractionation of the amylose has again occurred, short chains being preferentially leached at 70° whilst the larger ones are not able to diffuse out until the granular structure is further disrupted by dispersion. Similar results have been obtained by Schoch,[8] who suggested that leaching methods at 70° were inefficient for fractionation, since 50% of the amylose " retrograded " *in situ* in the granule. This appears unlikely, as *insolubilized* retrograded amylose cannot be redispersed at 98° under the conditions used here, and it is more probable that simple subfractionation occurs.

Aqueous Leaching and Granular Structure.—General comments on granular structure have been made previously.[1,5] Only the question of the nature of the granular " sacs " will be dealt with here. In agreement with Frey-Wyssling [9] and Meyer and Menzi,[10] we have found that potato starch readily forms granular " sacs " on treatment with hot water. Depending on the extraction temperature, these sacs contain from 88 to 98% of amylopectin, and retain (microscopically) the characteristics of swollen, disrupted granules. The persistent, highly swollen remains of granules, after relatively prolonged heating in water, suggest that strong secondary valency forces of the hydrogen-bond type must be present, *i.e.*, close and compact packing of the amylopectin must occur. Dissolution of amylopectin must simply entail disruption of these bonds. This problem is complex, and is being further examined.

Molecular Size of Potato Amylose.—The observed molecular size of any amylose depends on the methods of isolating and fractionating the starch.[1] In this work, where methods were chosen to avoid as far as practicable any hydrolytic degradation, the number-average $\overline{D.P.}$ of potato amylose has been found to be of the order of 4000 glucose residues. This is to be compared with previous values in the literature of 240—1130,[1] and emphasises the importance of oxygen-free conditions for the dispersion. (Preliminary unpublished experiments have shown that the presence of oxygen causes degradation.) In addition, this value for the molecular size was independent of the variety of potato. A value of about 4000 was found for both the Redskin and Arran Banner potato starches studied here, whilst a similar result has been obtained by Mr. W. A. J. Bryce for Majestic and Golden Wonder potato starches.

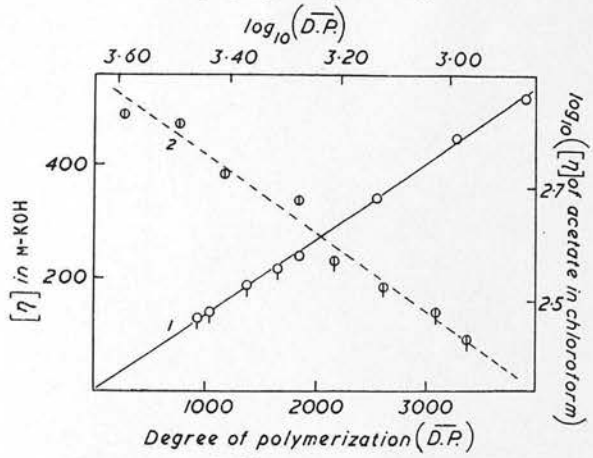
This value for the $\overline{D.P.}$ must be a *minimum*, since the possibility of inadvertent degradation in this work still cannot be eliminated.

Relation between $\overline{D.P.}$ and $[\eta]$ for Potato Amylose.—The results in this and previous work [5] enabled values to be calculated for K and α in the relation, $[\eta] = K(\overline{D.P.})^{\alpha}$. Although the distribution of molecular weight in some of the samples may well have been altered, it was found that, within experimental error, there was a linear relation between $[\eta]$ in M-potassium hydroxide and the $\overline{D.P.}$ derived from osmotic-pressure measurements

on the corresponding acetate. This relationship is illustrated in Fig. 2 (curve 1) which also includes the previously reported values for acid-degraded samples of amylose.[5] The equation, $\overline{\text{D.P.}} = 7{\cdot}4[\eta]$, holds for $\overline{\text{D.P.}}$ values up to 4000.

For values of $[\eta]$ for the acetates in chloroform solution, calculation by the method of least squares showed that the results were best represented by the equation, $[\eta] = 4{\cdot}30(\overline{\text{D.P.}})^{0{\cdot}61}$ (see Fig. 2, curve 2).

FIG. 2. *Graph of* $[\eta]$ *as a function of* $\overline{D.P.}$



(1) ($[\eta]$ *in* M-KOH) versus ($\overline{D.P.}$ *of acetate from osmotic-pressure measurements*).

○ *this work*; ○ *previous results* (*see ref.* 5).

(2) $\log_{10}$ ($[\eta]$ *of acetate in* $CHCl_3$) versus $\log_{10}$ (*D.P. of acetate from osmotic-pressure measurements*).

① *this work*; ① *previous results* (*see ref.* 5).

The significance of the above values of α in the modified Staudinger law, together with calculations of molecular dimensions, will be discussed elsewhere.

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DEPARTMENT OF CHEMISTRY, UNIVERSITY OF EDINBURGH. [*Received, February 25th*, 1957.]

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Physicochemical Studies on Starches. VII. The Infrared Absorption Spectrum of the Amylose-Iodine Complex

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INTRODUCTION

The amylose-iodine complex has been widely studied[1] and a linear arrangement of the iodine molecules in the internal channel of the amylose "helix" appears to be well established for the solid state. However, the nature of (*a*) the internal surface of the helix and (*b*) the amylose-iodine interaction is uncertain. In 1939, Freudenberg *et al.*[2] postulated that the pyranose rings are in a boat form (which in Reeves' convention[3] is of the type B1, Fig. 2), and that the iodine "dissolved" in the essentially paraffinic internal channel of the helix. More recently, Stein and Rundle[4] suggested a model in which no assumption was made about the conformation of the pyranose rings; the amylose-iodine interaction was ascribed to dipolar forces. Murakami[5] considers that this interpretation is inconsistent with West's x-ray measurements[6] of the periodicity of iodine in polymer-iodine complexes, and has suggested that charge-transfer interaction (similar to that postulated by Mulliken[7] for complexes of iodine with alcohols and ethers) occurs between iodine and the oxygen atoms in amylose. His model is based on a chair conformation in which one hydroxyl group and the glycosidic oxygen atoms lie on the internal surface of the helix. In both the Stein-Rundle and Murakami models, the complex is considered to be stabilized by resonance in the polyiodine chain.

Since iodine-oxygen interaction in solutions of iodine and iodine cyanide in ethers has been detected by infrared spectroscopy,[8,9] the infrared absorption spectra of amylose-halogen complexes was investigated in an attempt to decide between the two types of models. Although West[6] has reported that complex formation with iodine, bromine, and iodine bromide does not change the infrared spectrum of amylose, nylon, cellulose or polyvinyl alcohol, it has been pointed out[10] that small changes in the spectra of polysaccharides are often obscured by traces of water. The spectra of complexes of iodine, bromine, and iodine cyanide with carefully dried amylose were therefore measured, together with the iodine and iodine cyanide complexes of α- and β-Schardinger dextrins and of polyvinyl alcohol for comparison.

EXPERIMENTAL

Amylose, which had been prepared as the butan-1-ol complex from potatoes (*var.* Arran Banner) by Cowie[11] was dehydrated with butan-1-ol and then dried over phosphorus pentoxide for three weeks in vacuum at 80°C. This treatment reduced the intensity of the water band at 1645 cm.$^{-1}$ to only 10% of that of the 1025 cm.$^{-1}$ band in amylose. Complexes were formed by keeping the dried product for three days in an evacuated desiccator at 80°C. over iodine (AnalaR), bromine (AnalaR), or iodine cyanide (prepared from AnalaR iodine and potassium cyanide,[8] and resublimed). Samples of α- and β-Schardinger dextrins (kindly supplied by Dr. F. Cramer of Heidelberg University) and of polyvinyl alcohol (Grade RH 349) were similarly dried and treated with iodine and iodine cyanide. The colors (Table I) of the complexes obtained were unchanged even by prolonged drying in vacuum at 80°C.

TABLE I
Colors of Complexes Formed by Treating Solid Polymers with Halogen Vapor

	Iodine	Iodine cyanide	Bromine
Amylose	Blue-black	Brown-black	Dark brown
α-Schardinger dextrin	Buff	Buff	Not prepared
β-Schardinger dextrin	Buff	Buff	Not prepared
Polyvinyl alcohol	Blue-black	Light orange	Not prepared

The blue-brown iodine complex of α-Schardinger dextrin was prepared from aqueous solution by Cramer's method,[12] and dried as described above, but the analogous iodine cyanide complex could not be obtained.

The absorption spectra of Nujol mulls of the polymers and complexes were determined to ± 3 cm.$^{-1}$ in the range 2250–650 cm.$^{-1}$, using a Hilger H800 spectrophotometer with sodium chloride optics.

Scale models (1 A. = 1 in.) of amylose were constructed from 2 cm. plastic balls and 1/16 in. rods, assuming the bond lengths determined crystallographically for the pyranose ring in α-glucose by McDonald and Beevers.[13]

RESULTS AND DISCUSSION

The infrared spectra of amylose and of its iodine complex, both dried and equilibrated with water vapor, are shown in Figure 1. While the spectra of the damp samples are identical, the spectrum of the dried iodine complex shows small peaks at 1101 and 1052 cm.$^{-1}$, which are not present in dried amylose. The spectrum of the bromine complex is very similar, and a new peak also appears at 1101 cm.$^{-1}$ in the iodine cyanide complex. (In iodine cyanide and all the iodine cyanide complexes studied, the C≡N vibration band[14] appeared at 2166 cm.$^{-1}$. Since the cyanide group has a very high force constant (16.8×10^5 dynes cm.$^{-1}$ in iodine cyanide[15]) the absence of a frequency shift on complex formation is no evidence for lack of interaction

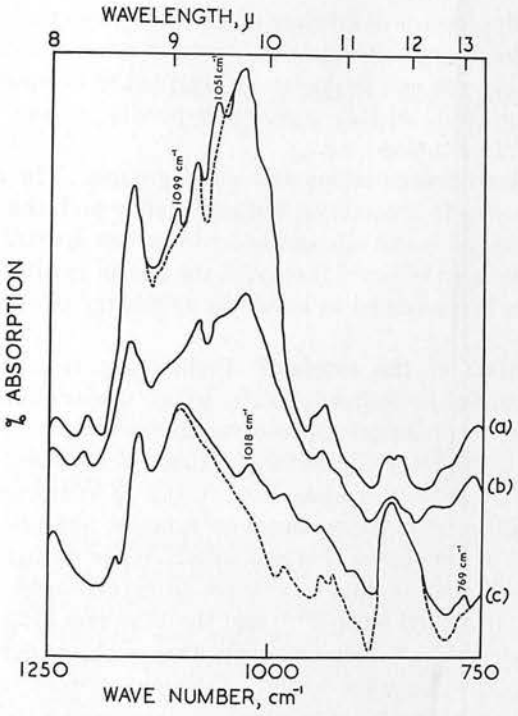

Fig. 1. Infrared absorption spectra of polymer-iodine complexes (full lines) and of original polymers (broken lines), displaced vertically for convenience. (*a*) Amylose (dried over phosphorus pentoxide at 80°C. in vacuum). (*b*) Amylose (equilibrated with water vapor); the spectra of the complex and of the parent polymer are identical. (*c*) Polyvinyl alcohol.

between the cyanide group and the polymer (*cf.* ref. 8). The same spectra were obtained after the amylose complexes had been shaken with chloroform or diethyl ether, and dried for several weeks in vacuum at 80°C. It would therefore appear that the changes in the spectra are due to complex formation[1,4] between amylose and the halogens, rather than to adsorption on the outside of the amylose helix.

No changes in spectrum were observed in the region 1750–650 cm.$^{-1}$ for the iodine and iodine cyanide complexes of α- and β-Schardinger dextrins. In the case of polyvinyl alcohol, although the spectrum of the iodine cyanide complex was unchanged from that of the original polymer, the iodine complex showed new peaks at 769 and 1021 cm.$^{-1}$ and a broadening of bands in the region 1000–900 cm.$^{-1}$ (Fig. 1c).

New peaks in the C—O stretching region[14] have been observed in solutions of iodine in diethyl ether[8,9] (1098, 1136 cm.$^{-1}$), dioxan[8] (1098 cm.$^{-1}$) and tetrahydrofuran[8] (1052 cm.$^{-1}$) and of iodine cyanide in dioxan[9] (1014, 1099 cm.$^{-1}$). These have been attributed to complex formation between iodine and the ethereal oxygen atom, and it is therefore suggested that the

new peaks in the spectra of amylose and polyvinyl alcohol complexes are also due to iodine-oxygen interaction.

Before the observed new peaks can be accepted as unequivocal evidence for complex formation of this type, other possible causes of changes in spectrum must be examined, *e.g.:*

(a) **Interaction between iodine and —CH groups.** The new peaks are unlikely to be due to interaction between iodine and the —CH groups in amylose, since no major changes in hydrocarbon spectra on treatment with iodine have been reported (except in the case of mesitylene, where the attached iodine is considered to lower the symmetry of the molecule,[8] *cf.* ref. 27).

(b) **Degradation of the amylose.** Preliminary results suggest that amylose is degraded by iodine bromide, iodine monochloride, and iodine trichloride, and by prolonged exposure to bromine vapor. The spectra of these complexes are markedly similar to those of acid-degraded amylose. However, sedimentation measurements in 0.2 *M* potassium hydroxide[16] indicate that little degradation occurs on complex formation with iodine.

(c) **Changes in the degree of crystallinity.** While changes in the region 1000–1150 cm.$^{-1}$ may be due to changes in crystallinity (*e.g.*, the 1144 cm.$^{-1}$ band in polyvinyl alcohol[17,18] and the 1140 and 1126 cm.$^{-1}$ bands[19] in nylon 66 and 610), it seems unlikely that such changes would occur merely on exposure to iodine vapor. Preliminary work on the effect of crystallinity on the absorption spectrum of amylose has indicated that no changes occur in this region of the spectrum.

In the absence of further evidence, the results reported here are compatible with Murakami's suggestion[5] that iodine-oxygen interaction occurs in the solid state. It is, of course, possible that such interaction also occurs in complexes for which no change of spectrum was observed.

Conformation of the Pyranose Ring in Amylose

If iodine-oxygen interaction occurs between a polyiodine chain and a surrounding amylose helix, the conformation of the pyranose ring must be discussed in terms of the forms which can be linked α:1,4′- such that oxygen atoms lie on the internal surface of the helix. The eight possible strainless ring forms[3] are shown in Figure 2 and the stereochemical positions of the substituents (other than hydrogen) are given in Table II.

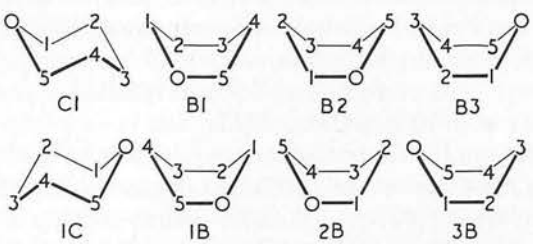

Fig. 2. The eight possible strainless conformations of the pyranose ring. The numerals represent carbon atoms, and the symbols follow Reeves' convention.[3]

TABLE II
Positions of Substituents in Pyranose Rings in Amylose

Conformation (Fig. 2)	Stereochemical position of large substituent[a]				
	C_1—O—	C_2—OH	C_3—OH	C_4—O—	C_5—CH_2OH
C1	↓	—	—	—	—
1C	—	↑	↓	↑	↓
B1	—	↓	—	—	—
1B	↑	—	↓	↑	↓
B2	↓	—	—	↓	↑
2B	—	↑	↓	—	—
B3	↓	↓	↑	↓	—
3B	—	—	—	—	↓

[a] Dashes indicate equatorial substituents, *i.e.*, those which project approximately into the plane of the pyranose ring. Arrows indicate axial substituents, which project above (↑) or below (↓) the plane of the ring, as drawn in Fig. 2.

The 1C, 1B, B2, and B3 conformations would be expected to be unstable, since two or more large substituents occur in *cis*-axial positions. Moreover, since substituents tend to favor equatorial positions,[20–22] the conformations C1, B1, and 3B, each with one axial substituent, might be expected to be more stable than the 2B conformation, with two *trans*-axial substituents. Subsequent discussion will be restricted to these three forms.

A detailed crystallographic study[13] has shown that α-glucose exists in the C1 form, and less complete x-ray data indicate the same conformation of the glucopyranose ring in sucrose[23,24] and cellulose[25] in the solid state.

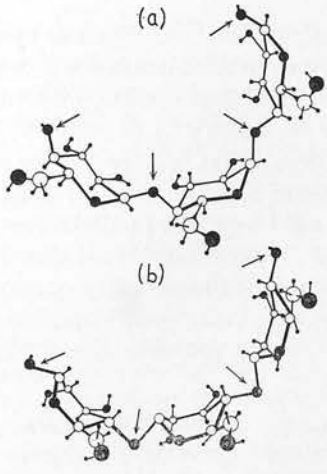

Fig. 3. Diagramatic representation of the effect of variations in glucopyranose ring conformation on the amylose helix. (*a*) Three C1 glucose units linked α:1, 4′. (*b*) Three B1 glucose units linked α:1, 4′. In each diagram: C = ○; O = ●; H = •. Substituents to the rings are shown with respect to orientation rather than size. Glycosidic oxygen atoms are indicated by arrows.

Moreover, Reeves' work on cuprammonium complexes suggests that this also holds for a number of simple glucosides in solution.[3] The C1 form was also postulated by Murakami[5] for the amylose-iodine complex, although the diagram given appears to represent the less stable 1C conformation. Scale models indicate that if glucopyranose rings in the C1 form are linked α:1,4′- such that substituents on analogous carbon atoms are on the same side of the glycosidic link, the glycosidic oxygen atoms and the C_2 hydroxyl group then lie on the internal surface of a helix (Fig. 3*a*). Rotation about the glycosidic link appears to be no more difficult than for models of amylose built up from pyranose rings in the B1 or 3B conformations (*cf.* ref. 26). The C1 helical structure would therefore appear to be compatible with iodine-oxygen interaction in the amylose-iodine complex.

The analogous polymer containing pyranose rings in the B1 configuration corresponds to Freudenberg's original model[2] of a helix in which the internal channel is essentially paraffinic (Fig. 3*b*); a scale model indicates that steric interaction between the substitutents in neighboring rings is somewhat greater in this structure than in the C1 helix. The 3B conformation, in which the axial substituent is the bulky —CH_2OH group, might be expected to be less stable than the B1 form in which a smaller hydroxyl group is axial. Moreover, if pyranose rings in the 3B form are linked α:1,4′- with all analogous substituents on the same side of the glycosidic bond, an essentially linear polymer results. Although a helix can be formed using a less regular arrangement of 3B rings, there appears to be considerable steric interaction between the substituents of adjacent rings, and the majority of glycosidic oxygen atoms do not lie on the surface of the helix. Thus neither the B1 or 3B conformation seems to fit the present results as well as the C1 form.

Although steric considerations and previous evidence suggest that the C1 conformation is the most stable form of the glucopyranose ring, Reeves[26] has explained the low reactivity of amylose with cuprammonium solutions in terms of a mixture of unreactive B1 and reactive 3B forms. While monomers in the C1 form would be expected[3] to react with the cuprammonium ion, in the case of a helical polymer with this conformation, the large $Cu(NH_3)_4^{2+}$ ions might have only limited access to the equatorial 2- and 3-hydroxyl groups, and the reactivity would thus be appreciably lowered. It would therefore appear that the solubility of amylose in cuprammonium solutions is not incompatible with the C1 form, which seems to be the most probable conformation of the pyranose ring in the solid amylose-iodine complex.

We wish to thank Professor E. L. Hirst, F.R.S. and Dr. F. J. C. Rossotti for their interest, Dr. D. M. W. Anderson for valuable discussions on infrared technique, and the Rockefeller Foundation for a grant.

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Synopsis

Changes in the infrared absorption spectrum of amylose on complex formation with iodine, bromine, and iodine cyanide vapor are consistent with the occurrence of halogen-oxygen interaction in the solid complexes. The implications of these results are discussed in terms of the possible conformations of the glucopyranose rings and their relationships to the helical structure for amylose. It is suggested that the most probable conformation is a chair-form in which only one substituent is axial to the plane of the ring.

Résumé

Les changements dans le spectra d'absorption infra-rouge de l'amylose par formation de complexe avec l'iode, le brôme et la vapeur de cyanure d'iode sont à mettre en rapport avec la formation de complexes solides par suite d'interactions halogène-oxygène. Les conséquences de ces résultats sont discutées sur la base des configurations possibles des anneaux glucopyranosiques et leurs rapports avec la structure hélicoïdale de l'amylose. On suggère que la configuration la plus probable est une forme-chaise, dans laquelle un seul substituant est axial par rapport au plan de l'anneau.

Zusammenfassung

Änderungen im infraroten Absorptionsspektrum von Amylose bei Komplexbildung mit Jod, Brom und Bromcyanid-Dampf sind gleichzeitig mit dem Auftreten von Halogen-Sauerstoff-Interaktion in den festen Komplexen feststellbar. Die Bedeutungen

dieser Resultate werden in Bezug auf die möglichen Konfigurationen der Glucopyranose-Ringe und ihre Beziehung zur schneckenförmigen Struktur für Amylose diskutiert. Es wird vorgeschlagen, dass die wahrscheinlichste Konfiguration eine Stuhl-Form ist, in welcher nur ein Substitutionsmittel zur Ebene des Ringes achsenförmig ist.

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continued from inside front cover

LETTERS TO THE EDITORS

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932. *Physicochemical Studies on Starches. Part VIII.* Further Observations on the Fractionation of Potato Starch.*

By J. M. G. Cowie and C. T. Greenwood.

The fractionation conditions necessary to achieve effective separation and minimise degradation of the components of potato starch have been critically examined. Laboratory-prepared and *commercial* starch samples have been compared. The amylose from commercial starch is much smaller ($\overline{D.P.}$ ~2500 glucose residues) than that from the laboratory-prepared sample ($\overline{D.P.}$ ~4000), and the effect of aqueous leaching at 70° and 98° also differs. The uniformity of structure of amylose in the granule is discussed. "Sub-fractions" obtained during the recrystallisation of the fractionation products of the laboratory-prepared starch have also been studied.

In Part VI of this Series,[1] we reported the results of aqueous leaching at various temperatures on laboratory-prepared potato-starch granules. The problem of fractionation and the fine structure of the starch granule are further considered here. It is essential to obtain the separated components in an unmodified form, and artefacts or degradation products must be avoided. Amylose is susceptible to oxidative degradation at elevated temperatures.[2] The extent to which this occurs during fractionation has now been examined in detail, as have methods suggested [3] for minimising this effect. Commercial potato starch has also been fractionated. In addition, the "sub-fractions" obtained during recrystallisation of the amylose component of laboratory-prepared starch have been studied in an attempt to establish the presence of material intermediate in structure between amylose and amylopectin.

Experimental

Preparation of Starch.—Starch was extracted from potatoes (var. Golden Wonder) by the method outlined previously.[4] A sample of commercial potato starch ("Superfine Farina") was kindly supplied by Messrs. Brown & Polson, Ltd.

Fractionation Methods.—The methods of fractionation were as shown in Table 1.

Characterisation of Fractionation Products.—Measurements of (*a*) iodine affinity (I.A.),[5] (*b*) limiting viscosity number $[\eta]$ in M-potassium hydroxide,[4] and (*c*) sedimentation velocity in 0·2M-potassium hydroxide [6] were carried out on the separated components as detailed previously.

Results and Discussion

Our previous results [1] have shown that amylose isolated from laboratory-prepared potato starch possesses a number-average degree of polymerisation ($\overline{D.P.}$) of the order of 4000 glucose units. This value was therefore taken as a standard to judge the effect of modifications in fractionation techniques.

Oxidative Degradation.—Many investigators have suggested that starch, particularly the linear amylose component, may be degraded during fractionation procedures.[3] By measuring the molecular weight of the separated components, we have shown that the presence of oxygen during fractionation is, in fact, a serious source of degradation. The results in Table 1 (cf. F1 and F2) show that the $\overline{D.P.}$ of the amylose produced by passage of oxygen through the fractionation and recrystallisation media is reduced by one half (equivalent to *ca.* 1·2 bonds broken/initial amylose molecule). Even when the recrystallisations are carried out under nitrogen, degradation is still appreciable. Amylopectin is also depolymerised under these exaggerated conditions.

* Part VII, *J. Polymer Sci.*, 1957, in the press.

Aqueous leaching of the commercial starch at 70° also gave amylose of $\overline{\text{D.P.}}$ 2400, and this value was unchanged when the granule was subsequently dispersed at 98° (see Table 1). This behaviour is completely different from that of laboratory-prepared samples,[1] and suggests that in the commercial samples all the amylose is equally accessible.

Uniformity of Structure of Amylose.—Our aqueous-leaching experiments [1] suggest that in potato starch there may be two amylose fractions, (1) easily accessible material of relatively low $\overline{\text{D.P.}}$ and (2) a fraction of higher $\overline{\text{D.P.}}$ requiring disruption of the granule before isolation. There is the possibility that these fractions may also differ in *structure*.[3] Although physical evidence shows that their iodine affinities are identical, and the same ln M *versus* ln $[\eta]$ relationship holds within experimental error, the $\overline{\text{D.P.}}$'s are so large that chemical methods are not satisfactory. However, β-amylase will degrade a linear amylose molecule completely to maltose, whilst its hydrolytic action stops at any branch-points or other anomaly.[3] Experiments carried out in collaboration with Mr. I. D. Fleming showed that the β-amylolysis limits of the different amylose fractions varied considerably (Table 2).

For laboratory-prepared starches, the complete degradation of the amylose leached at 70° suggests that this short-chain material is completely linear. Extraction at higher temperatures gives amylose which is incompletely hydrolysed, the amount of resistant material increasing with increase in temperature and consequent disruption of the granule. The probable nature of this barrier to β-amylolysis will be discussed elsewhere, but it appears to be associated with disruption of the granule and hence some branching is not improbable.[3]

The leaching and β-amylolysis results for the commercial starch differ greatly. All the amylose products were of virtually the same size, and, whilst that leached at 70° was again linear, the product of a conventional dispersion was also nearly completely hydrolysed.

Analysis of the "Sub-fractions" obtained during Fractionation.—The granule may contain fractions with properties intermediate between those of amylose and amylopectin.[3] In an attempt to identify such material, a careful analysis was made of all the products from a fractionation of a laboratory-prepared starch, especially the solids from the mother-liquors obtained from recrystallisation of amylose. The results are shown in Table 3. The weight of material in the supernatant liquors decreased regularly and the iodine affinity increased generally. With the exception of the solid from the supernatant liquor from butanol complex 2, the iodine-binding curves were identical in character with those previously reported for a similar analysis of *Hevea brasiliensis* starch.[7] Examination of the thymol complex in the ultracentrifuge revealed an apparently *homogeneous* fraction, although it contained about one-third of amylopectin. Amylopectin normally has a sedimentation constant about twenty times larger than amylose, but the amylopectin product isolated on reprecipitation of the thymol complex had a value equivalent to that for amylose itself. Without further evidence, it is impossible to decide whether there are also two amylopectin fractions of widely differing $\overline{\text{D.P.}}$, or whether this "thymol-amylopectin" has a different structure, although it has similar iodine-binding properties to amylopectin. Experiments are in progress to investigate this. Recrystallisation of the amylose apparently results in the elimination of branched material and presumably also short-chain amyloses, although the latter are difficult to detect by potentiometric iodine titration. With the possible exception of the "thymol-amylopectin," no major component has been detected which might be an intermediate between amylose and amylopectin.

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135. *Physicochemical Studies on Starches. Part IX.* The Mechanism of the β-Amylolysis of Amylose and the Nature of the β-Limit Dextrin.*

By J. M. G. Cowie, I. D. Fleming, C. T. Greenwood, and D. J. Manners.

The mechanism of the hydrolysis of amylose by (*a*) pure β-amylase, and (*b*) β-amylase and Z-enzyme, has been studied by measuring the $\overline{\text{D.P.}}$ of the residual polymer at varying degrees of conversion into maltose. Amylose fractionated severally in presence and absence of air, and a sub-fraction obtained by aqueous leaching of the granule, have been used as substrates. The molecular properties of the various 50% conversion dextrins and the 77% β-limit dextrins were examined in detail. In all cases, hydrolysis proceeded by an essentially single-chain mechanism as there was no evidence of molecules other than amylose of $\overline{\text{D.P.}} \geqslant$ the original and maltose in the digest. The structure of the β-limit dextrin, which is thought to contain a randomly-situated barrier to β-amylolysis, is discussed.

The action of β-amylase on amylose, which commences at the non-reducing end of the molecule, involves hydrolysis of alternate α-1 : 4-glucosidic linkages with the production of maltose. In a recent paper,[1] we reported the β-amylolysis of an amylose of high molecular weight, prepared [2] by thymol fractionation of potato starch after complete disruption of the granules. Our results confirm that the *pure* enzyme degrades only *ca.* 75% of amylose, and that for complete conversion into maltose a second enzyme (Z-enzyme) is required. The specificity of the latter enzyme was examined, and evidence presented that the β-amylolysis limit for the pure amylose is not an artefact associated with the colloidal instability of the amylose substrate.[1] The nature of the barrier to β-amylolysis is not known.

The specific mode of action of the β-amylase has been in dispute.[3] Maltose may be produced by the enzyme either by (1) attachment to one amylose molecule and then, by stepwise removal of maltose units, complete degradation before attack on another amylose molecule (" single-chain " action), or (2) by removal of one maltose unit on each random collision with an amylose molecule, with the result that all chains in the system will be shortened simultaneously (" multi-chain " action). Reaction mechanisms between (1) and (2) are also possible. However, determination of the molecular weight of the residual polysaccharide at intermediate stages of β-amylolysis will indicate which mechanism is operative. Under normal experimental conditions, with a large substrate : enzyme ratio, the number-average degree of polymerisation ($\overline{\text{D.P.}}$) of the residual amylose at any time during a single-chain reaction will be the same as that for the original up to the stage when the number of substrate molecules is approximately equal to the number of enzyme molecules (at this point, a multi-chain mechanism is inevitable). For multi-chain action throughout, the $\overline{\text{D.P.}}$ of the amylose will decrease as the reaction proceeds, the reduction being proportional to the percentage conversion into maltose. Experiments of this type have been carried out by Kerr and Cleveland,[4] who found that the polymeric product isolated at about 50% conversion into maltose possessed virtually the same iodine affinity, limiting viscosity number, and $\overline{\text{D.P.}}$ as the original amylose. In our work (a preliminary account of which has appeared [5]), we have extended this type of experiment to include the measurement of the $\overline{\text{D.P.}}$ of the residual polymer at varying degrees of conversion into maltose resulting from the action of (*a*) pure β-amylase, and (*b*) β-amylase and Z-enzyme. Amylose fractionated severally in presence and absence of air, and a subfraction obtained by aqueous leaching of the granule, have been used as substrates. The molecular size of various 50% conversion dextrins and 77% β-limit dextrins has been examined, and the structure of the β-limit dextrin is discussed.

* Part VIII, *J.*, 1957, 4640.

Experimental

Preparation of Amylose Samples and their Characterisation.—Potato starch (var. Arran Banner) was fractionated by (1) dispersion in the presence or absence of oxygen, and (2) aqueous leaching at 70°. These methods and those used to characterise the polymers have been described in detail previously in this Series.

Preparation of Enzymes.—Barley β-amylase and soya-bean β-amylase were used. Their preparation and properties have been described elsewhere.[1]

Digest Conditions.—At pH 4·6 and 35° soya-bean β-amylase showed no Z-enzyme activity and converted 77% of amylose samples of high $\overline{\text{D.P.}}$ into maltose, whilst under these conditions barley β-amylase hydrolysed all samples completely.[1] Amylose was dissolved directly in water from the well-centrifuged butan-1-ol complex and buffered with acetate to pH 4·6. Enzyme solution [1] was added and the reaction rate followed by withdrawal of aliquot parts at intervals and estimations of the liberated maltose. In all digests, the concentration of enzyme (100 units per mg. of amylose) was such that 50% conversion had occurred within 30 min., and hence retrogradation of amylose was unlikely.

Isolation of and Measurements on β-Amylolysis Products.—The $\overline{\text{D.P.}}$ of the polymeric product at different percentage conversions was obtained by withdrawing aliquot parts (2 ml.) of the digest and adding M-potassium hydroxide (0·5 ml.). The resultant 0·2M-potassium hydroxide solution (maximum concentration of amylose, 0·18 g./100 ml.) was examined directly in the Spinco ultracentrifuge. Each aliquot portion was studied at three dilutions; the limiting dilution was 0·02 g./100 ml. The 50% conversion and 77% β-limit dextrins were isolated by adding butan-1-ol, then heating the digest for 2 min. on a boiling-water bath (to complete deactivation of enzyme) and allowing the butan-1-ol complexes to be precipitated at room temperature during 24 hr. After removal by centrifugation, the complexes were thoroughly washed with butan-1-ol-saturated water to remove maltose.

Discussion

A study of the β-amylolysis of amylose is complicated by the fact that the β-limit depends on the method of preparation of the amylose. Our aqueous leaching experiments [1,2,6] have shown that potato starch granules contain an easily accessible amylose fraction of relatively low molecular weight, which is completely hydrolysed by pure β-amylase. In this work, the action pattern of β-amylase on the *whole* amylose has been studied in order to investigate the β-limit dextrin and also to use conditions equivalent to those of other workers. The action pattern under these conditions proved to be identical with that for the completely linear amylose prepared by aqueous leaching.

Action of Pure β-Amylase.—(a) *Whole amylose.* Preliminary trial digests showed that the sample of amylose used ($\overline{\text{D.P.}}$ 3200) gave 77% conversion into maltose. When aliquot parts were removed at intervals, and studied in the ultracentrifuge, the sedimentation constant (S_{20}) of the residual polymer as a function of the concentration (c) was as shown in Fig. 1*a*. (The sedimentation constant for amylose is concentration-dependent, as previously reported.[7]) All the points lie on the same curve, within experimental error, although there is a tendency for the values in the earlier stages of conversion to be slightly higher. Fig. 1*a* also shows the corresponding plot of S_{20} against $S_{20}.c$ as recommended by Gralén [8] to facilitate extrapolation to infinite dilution. The points again lie on the same curve. The limiting value of S_{20} for all the residual amyloses was therefore independent of the degree of conversion into maltose up to and including the 77% limit. This result would not be expected on the basis of multi-chain action.

In order to confirm that the sedimentation constant of the β-limit dextrin was unchanged, it was isolated from a large-scale digest. A comparison of the properties with those for the original amylose were as shown:

	$[\eta]$	Iodine affinity	$10^{13}(S_{20})_0$
Original amylose	430	19·5	13·1
77% limit	415	19·2	13·1

The sedimentation results are shown in Fig. 1*b*. The agreement in sedimentation constants shows that the liberated maltose does not influence S_{20} when portions of the digest are

studied directly. The properties of the two polymers are identical within experimental error.

Further, paper chromatography of the digest showed that sugars other than maltose were not present. It was apparent that, in the hydrolysis of amylose by pure β-amylase at pH 4·6 and 35°, the hydrolysate contains only amylose with a $\overline{D.P.}$ greater than or equal to that of the original and maltose. Multi-chain action is therefore excluded.

If amylose is attacked only from the non-reducing end then, as has been stressed by Kerr and Gehman,[9] the rate of maltose production should be proportional to the molarity rather than the actual weight concentration of amylose. Accordingly solutions of amylose of varying $\overline{D.P.}$ but equal molarities should show the same rate of production of maltose. Results of experiments for two $1{\cdot}4 \times 10^{-6}$M-solutions were as shown:

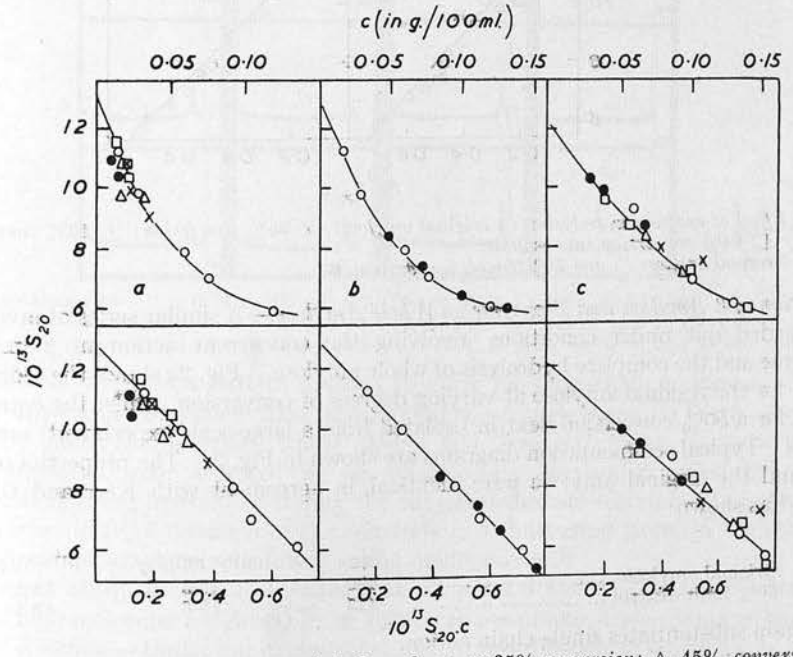
Conversion (%) into maltose at a given time (min.).

$\overline{D.P.}$ of amylose	5	10	15	30	60
3200	34·9	44·9	55·6	71·0	77·8 (const.)
2000	32·5	44·7	53·0	71·1	75·2 (const.)

These results again substantiate an essentially single-chain action. Similar results have been reported by Kerr and Gehman.[9]

Although it has been reported[10] that oxygen-treatment can introduce barriers to the phosphorolysis of amylose, it was shown elsewhere[1] that amylose prepared in the presence

FIG. 1. S_{20} versus c *and* S_{20} versus $S_{20}.c$ *for amyloses treated with pure β-amylase.*



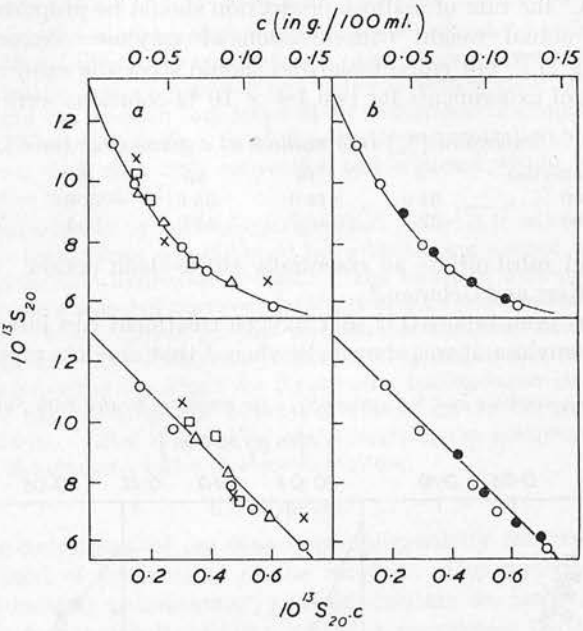
(*a*) *Effect of enzymic hydrolysis:* ○ *original amylose;* × 35% *conversion;* △ 45% *conversion;* □ 55% *conversion;* ● 77% *conversion into maltose.*
(*b*) *Original amylose* ○ *and 77% limit dextrin* ●.
(*c*) *Effect of enzymic hydrolysis (oxygen-treated amylose):* ○ *original amylose;* × 23% *conversion;* △ 34% *conversion;* □ 56% *conversion;* ● 75% *conversion into maltose.*

of oxygen was hydrolysed as far as 75% conversion into maltose. The enzymic degradation of this amylose sample was also studied in detail. Fig. 1*c* shows the plots of S_{20} against c and against $S_{20}.c$ for the residual amylose at varying degrees of conversion into maltose. Again, within experimental error, the limiting value of S_{20} for the residual amylose is independent of the conversion up to, and including, the limit. Typical

sedimentation diagrams are shown in Fig. 3. Essentially single-chain attack is therefore again established.

(b) *Aqueous-leached amylose.* To confirm the above action pattern, a 50% conversion product was prepared from a sample of amylose leached [2,6] at 70°. The limiting viscosity number of this product was the same as that for the original amylose, within experimental error (for the original amylose, $[\eta] = 270$; for the 50% conversion dextrin, $[\eta] = 265$).

FIG. 2. S_{20} versus c *and* S_{20} versus $S_{20}.c$ *for amyloses treated with* β*-amylase and Z-enzyme.*



(*a*) *Effect of enzymic hydrolysis*: ○ *original amylose*; × 35% *conversion*; △ 53% *conversion*; □ 64% *conversion into maltose.*
(*b*) *Original amylose* ○ *and* 50% *conversion dextrin* ●.

Action of β*-Amylase and Z-enzyme on Whole Amylose.*—A similar series of investigations was carried out under conditions involving the concurrent action of β-amylase and Z-enzyme and the complete hydrolysis of whole amylose. Fig. 2*a* shows the sedimentation results for the residual amylose at varying degrees of conversion, whilst the corresponding results for a 50% conversion dextrin (isolated from a large-scale experiment) are shown in Fig. 2*b*. Typical sedimentation diagrams are shown in Fig. 3. The properties of the 50% limit and the original amylose were identical, in agreement with Kerr and Cleveland's results,[4] as shown:

	$[\eta]$	Iodine affinity	$10^{13}(S_{20})_0$
Original amylose	430	19·5	13·1
50% limit dextrin	425	19·6	13·1

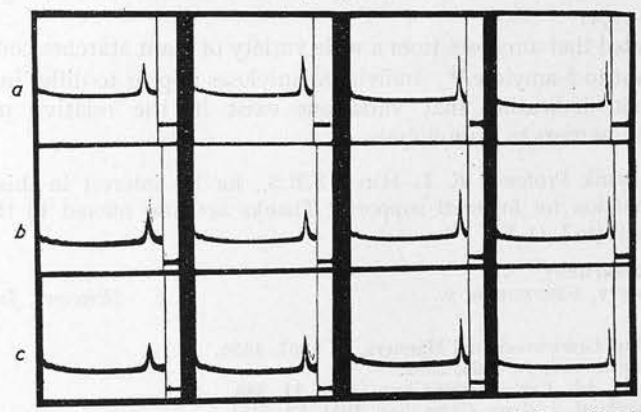
This again substantiates single-chain action.

Effect of β*-Amylase Action on the Absorption Spectra of the Amylose–Iodine Complex.*—Swanson [11] observed that the wavelength of maximum absorption ($\lambda_{max.}$) of the amylose–iodine complex was unaltered during β-amylolysis, thus indicating single-chain action. However, Bourne and Whelan [12] have criticised Swanson's iodine-staining conditions, and found a movement of $\lambda_{max.}$ from 660 to 580 mμ when equal weights of polysaccharide were stained during the formation of 86% of maltose. We have therefore incubated amylose of $\overline{\text{D.P.}}$ 3200 with β-amylase, and stained equal weights of residual amylose at intervals up to the production of 84% of maltose. In all samples, the $\lambda_{max.}$ remained unchanged at *ca.* 660 mμ. It now seems probable that Bourne and Whelan's results are due, in part, to

contamination of amylose with amylopectin (the β-dextrin of amylopectin [13] has $\lambda_{max.}$ *ca.* 540 mμ), and are not the direct result of multi-chain action. (Amylose prepared recently in this Laboratory by the aluminium hydroxide method [14] contained only 81—87% of amylose.) It should also be noted that iodine-staining measurements on amylose of high $\overline{D.P.}$ (*ca.* 3000) do not enable the reaction mechanism to be determined. (With single-chain action, no movement of $\lambda_{max.}$ is to be expected, whilst, with multi-chain action, the residual amylose at 84% β-amylolysis would have a $\overline{D.P.}$ of *ca.* 500; $\lambda_{max.}$ for this would be little altered.[3])

The Action Pattern of β-Amylase.—All the above results are inconsistent with the concept of multi-chain action. Rather, it appears that, under the conditions of our experiments, the amylose after making contact with a substrate molecule hydrolyses it completely before attacking another molecule, in agreement with Kerr and Cleveland's results.[4] This action is consistent with the remarkably high "turn-over number" (250,000) reported [15] for the enzyme.

FIG. 3. *Tracings of typical sedimentation diagrams. Schlieren wire assembly. In all cases, speed = 60,000 r.p.m.; movement is from right to left; times given are those after reaching full speed; the figures in parentheses after the times indicate the angle of the Schlieren wire.*



(*a*) *Original amylose*: c = 0·14 g./100 *ml. at* 5 (70°), 9 (65°), 13 (60°), *and* 17 (60°) *min.*
(*b*) 77% *limit dextrin*: c = 0·13 *g./100 ml. at* 5 (70°), 9 (60°), 13 (60°), *and* 19 (60°) *min.*
(*c*) 50% *conversion dextrin*: c = 0·11 *g./100 ml. at* 5 (70°), 9 (60°), 14 (60°), *and* 18 (60°) *min.*

Nevertheless, the action pattern appears to differ for short-chain amyloses. Recent studies by Bird and Hopkins [16] have shown that amylose-dextrins ($\overline{D.P.}$ 16—30) were degraded by multi-chain action, whilst Bailey and French [17] found that short-chain synthetic amyloses were attacked by an intermediary mechanism, whereby several glucosidic linkages are hydrolysed during the enzyme–substrate reaction. It is probable that the relative rate of diffusion of the substrate is a controlling factor in the reaction, since at higher temperatures multi-chain action predominates.[18]

The present study therefore indicates that, at pH 4·6 and 35°, β-amylase degrades amyloses of high molecular weight ($\overline{D.P.} \approx 10^3$) by an essentially single-chain mechanism.

Order of Reaction.—Under our experimental conditions, the rate of reaction was so fast that a detailed analysis was not possible. However, the reaction in its initial stages was not of a definite zero or first order (cf. refs. 19 and 20), but the value of k altered. For the overall reaction, the plot [21] of $1/\overline{D.P.}$ against t was not linear. This reaction is being investigated further.

Nature of the β-Limit Dextrin and the Structure of Amylose.—As indicated above, amylose in potato starch is heterogeneous both in $\overline{D.P.}$ and in behaviour on β-amylolysis. Our previous results indicate that there is 30—40% of amylose of $\overline{D.P.}$ 1800, which is completely hydrolysed to maltose by *pure* β-amylase. The sample of whole amylose used in this work had a $\overline{D.P.}$ of 3200, and a β-limit of 77%. It therefore follows that the $\overline{D.P.}$ of the

presumably incompletely hydrolysed amylose is of the order of 6000, and that, to the first approximation, it has a β-limit of 50%. (This accounts for a final $\overline{D.P.}$ of *ca.* 3000.) A 50% limit suggests that the barrier to β-amylolysis is randomly distributed throughout the high-molecular-weight amylose.

Although the nature of the barrier has not been established, several possibilities have been considered. The barrier may be situated in the main amylose chain itself, or in a side-chain joined through position 2, 3, or 6 of a constituent glucose residue in the main chain. The former possibility would imply that phosphorylase is not completely specific for α-1 : 4-linked glucopyranose residues.

A side-chain formed by an ester-phosphate group is unlikely, since bone phosphatase, which dephosphorylates starch, does not remove the anomalous linkage.[22] Further, the suggestion by Peat and his co-workers [22] that single glucose residues are attached to a main amylose chain could not be verified experimentally by Hopkins and Bird.[23] An alternative possibility is that the molecule is branched, each branch containing several hundred glucose residues. Kerr and Cleveland [24] have, in fact, suggested that potato and tapioca amylose are singly branched, and contain 1—3 branches per molecule. Our previous studies [1] suggest that, if branching occurs, the interchain linkage is not of the α-1 : 3- or α-1 : 6-type.

It must be noted that amyloses from a wide variety of plant starches contain anomalies which are resistant to β-amylase.[25] Individual amyloses appear to differ in both $\overline{D.P.}$ and β-amylolysis limit, indicating that variations exist in the relative proportion and distribution of the barriers to β-amylolysis.

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136. *Physicochemical Studies on Starches. Part X.* The Molecular Weight of the Water-soluble Polysaccharides of Sweet Corn,* Zea mays.

By C. T. Greenwood and P. C. Das Gupta.

Detailed results are given of physical studies on samples of the water-soluble glucosans of sweet corn, *Zea mays*, isolated by Peat, Whelan, and Turvey.[1] Another sample of the total water-soluble polysaccharide has also been prepared. The glucosan-product had a very high molecular weight, and, further, on dissolution and fractionation with 67% acetic acid gave only 2% of the total as " soluble " material. It is therefore suggested that in the sample of seed examined the water-soluble glucosan is essentially *homogeneous*, and has a molecular weight of the order of 30×10^6, and an average length of unit chain of 14. The structure of the glucosan is discussed in relation to that of glycogen and amylopectin.

Recently, Peat, Whelan, and Turvey [1] carried out a comprehensive study of the water-soluble amylaceous polysaccharides of sweet corn (*Zea mays*), in which they established the conditions necessary to avoid extensive enzymic degradation of polysaccharide during isolation. A full review of the previous literature was also given. In this paper, we report details of the physical measurements carried out on the samples kindly placed at our disposal by Professor S. Peat, together with the results of our own investigations on the isolation and fractionation of *Zea mays* glucosans.

Experimental

All samples were dried at 80° *in vacuo* before analysis. The carbohydrate content of the polysaccharide was determined by acid hydrolysis (0·5N-sulphuric acid for 7 hr. at 98°), followed by the estimation of the amount of liberated glucose by alkaline ferricyanide.[2] Nitrogen estimations were by the semimicro Kjeldahl method.

Isolation of the Water-soluble Polysaccharides of Zea mays.—Mature *Zea mays* (*var.* Golden Bantam; 400 g.) was ground and extracted with 0·01N-mercuric chloride as described by Peat *et al.*[1] Extracts were filtered through muslin, and the starch allowed to settle before the supernatant liquors were passed through the Sharples supercentrifuge and concentrated to $\frac{1}{10}$ vol. (under reduced pressure at 30°). Coagulated protein and any other insoluble material were then removed by careful filtration, and the soluble polysaccharide precipitated by the addition of ethanol (1·5 vol.) at 0°. The precipitate was removed on the centrifuge, washed with acetone and ether and dried (yield 79 g.) (Found: N, 0·32; carbohydrate, 88·8%.) The sugar-containing supernatant liquid was treated as by Peat *et al.*,[1] to yield approximately 13 g. of material, which contained no polysaccharide disclosed by chromatography.

Fractionation of the Water-soluble Polysaccharide.—Water-soluble polysaccharide (22 g.) was dissolved in water (300 ml.) and cooled to 0°, and glacial acetic acid (600 ml.) was added slowly with stirring, the temperature being kept *at* 0° *during the whole period.* After the mixture had been then kept at −2° for 18 hr., the precipitated phytoglycogen-A was removed on a refrigerated centrifuge (at 0°), and washed well with acetone to remove the acid. (Isolation of the material by centrifugation at room temperature caused slightly more degradation, as shown by a decrease in sedimentation constant from 226*s* to 214*s*.) After dissolution in water, the pH was adjusted to 6·5 with sodium hydroxide before reprecipitation of the polysaccharide by the addition of ethanol (1·5 vol.). The product was washed with ethanol and with ether and dried (yield 20·5 g.) (Found: N, 0·2; carbohydrate, 92%). The phytoglycogen-B was isolated from the original supernatant liquor by the addition (at 0°) of ethanol (0·5 vol.) (yield 0·83 g.) (Found: N, 1·0; carbohydrate, 47%).

Attempted Removal of Protein and Fractionation of the Water-soluble Polysaccharide.—Polysaccharide was dissolved in 0·1M-sodium chloride (8% solution) and shaken overnight with toluene ($\frac{1}{10}$ vol.). The toluene layer was then allowed to separate and was removed. The process was repeated until the amount of coagulated material in the toluene layer was negligible.

* Part IX, preceding paper.

The solution was then cooled to 0°, divided into 3 portions, and treated as follows: (i) the polysaccharide was precipitated with ethanol (1·5 vol.), washed with ethanol and with ether, and dried (Found: N, 0·09%); (ii) phytin was removed by acidifying the solution with hydrochloric acid (1%) and precipitating the polysaccharide immediately; [1] the polysaccharide was then isolated after adjustment of the pH of the solution to 6·5 as above; (iii) phytoglycogen-A and -B was obtained by the addition of glacial acetic acid (2 vol.) as above; phytoglycogen-A was purified from phytin and adhering acid; again the yield of phytoglycogen-B was small, and it was difficult to isolate this material by precipitation.

Characterization of the Polysaccharide Fractions.—Measurements of (i) average length of unit chain by periodate oxidation, (ii) iodine affinity, and (iii) sedimentation velocity in 0·2M-sodium chloride were carried out as described in previous papers in this Series. The sedimentation constant (S_{20}) for the polysaccharides was found to be the same in water and in 0·2M-aqueous sodium chloride, and measurements have been made in both solvents. S_{20} was a linear function of c; its dependence on concentration is expressed here by values of k in the equation $S_{20} = (S_{20})_0(1 - kc)$, where $(S_{20})_0$ is the sedimentation constant at infinite dilution.

Determination of Diffusion Coefficient (with W. A. J. Bryce).—The diffusion coefficient of the polysaccharides dissolved in 0·2M-sodium chloride was measured in a new type of diffusion cell designed for the Antweiler microelectrophoresis and diffusion apparatus. In this instrument, the refractive-index gradient curve is measured by means of a Jamin interferometer. Diffusion coefficients (D_m) were calculated by the moment method. Measurements were carried out at 20°. Details of the apparatus and method will be presented elsewhere.

Light-scattering Measurements (with I. G. Jones).—Measurements were made with a Brice–Phoenix Photometer (Model 1000D), with cylindrical cells and the narrow diaphragm system.[3] Turbidities (τ) were determined by the "working standard" method. The dissymmetry ratio ($I_{45°}/I_{135°}$; corrected for back-reflection) was also measured. Solvent (0·1M-sodium chloride) was filtered through sintered glass (G5) directly into the scattering cell. Polysaccharide solutions (approx. 0·1%) were filtered through sintered glass (G4), and successive aliquot parts of the clarified solution were added to a weighed amount of solvent in the scattering cell to form a concentration series in the range (1—20) $\times 10^{-5}$ g./ml. [Concentrations (c in g./ml.) were estimated by hydrolysing a portion of the original filtered solution, and estimating the amount of liberate dglucose by the alkaline ferricyanide–ceric sulphate method.[2]] This procedure gave solutions which showed reproducible turbidities, and, on the whole, reproducible low dissymmetries. (Large dissymmetries occasionally observed were thought to be due to anomalous aggregates which were not removed by filtration.) Molecular weights (M) were calculated from the equation:

$$Hc/\tau = 1/M(P_{90°}) + 2B[redacted]c/\boldsymbol{R}T \text{ where } H = 32\pi^2n^2(\mathrm{d}n/\mathrm{d}c)^2/3\lambda^4N;$$

($P_{90°}$) is the particle scattering factor, and was calculated from the dissymmetry on the assumption that the molecules are spherical; [4] B is a constant. The refractive index increment ($\mathrm{d}n/\mathrm{d}c$) was measured at 546 mμ on a Brice–Phoenix differential refractometer,[5] and a value of 0·146 was

TABLE 1. *The properties of Peat, Whelan, and Turvey's polysaccharides.*[1]

Polysaccharide	Extraction [a]	Carbohydrate (%) [b]	Unit chain length [b]	Slope of iodine titration [c]	k [d]	$10^{13}(S_{20})_0$ [e]	10^7D_m	$10^{-6}M$
Phytoglycogen A	+	94·2	13·2	0·017	—	(185)	—	(20)
,, ...	—	95·1	9·7	0·007	—	(55)	—	(6)
,, B	+	94·7	7·3	0·007	—	(48)	—	(4)
,, ,, ...	—	93·8	5·8	0·000	—	(2·3)	—	—
Fractions								
55—60% AcOH ppt.	+	—	10·3	—	0·07	146	0·6	15
60—65% ,,	+	—	11·0	—	0·09	131	—	(14)
65—70% ,,	+	—	9·7	—	0·15	78	—	(8)
>70% ,,	+	—	7·8	—	0·75	9	3·5	0·2

[a] Extraction with (+) or without (−) $HgCl_2$. [b] Values from ref. 1. [c] Slope of linear portion of iodine titration curve. Values from Part III.[6] [d] Value of k in the equation $S_{20} = (S_{20})_0(1 - kc)$, where c = g./100 ml. [e] Values in parentheses were determined at a concentration of 0·5 g. per 100 ml. [f] Calc. from $M = \boldsymbol{R}T(S_{20})_0/(1 - \overline{V}\rho)D_m$, where $\overline{V}$ = partial specific volume = 0·62, the value found for glycogen (unpublished results). For the values in parentheses, D_m has been assumed to be $0·6 \times 10^{-7}$.

TABLE 2. *The properties of the polysaccharides isolated in this work.*

	Total polysaccharide *		Phytoglycogen A *		Phytoglycogen B *
	(*a*)	(*b*)	(*a*)	(*b*)	(*b*)
Carbohydrate (%)	92	100	92	97	83
Length of unit chain	14·0	14·0	13·0	13·0	11·3
k †	0·025	0·025	0·025	—	0·75
$10^{13}(S_{20})_0$	252	242	226	—	8
$10^7 D_m$	—	0·53	—	—	—
$10^{-6}M$	(30) ‡	29	(27) ‡	—	(0·14) ‡

* (*a*) Purified free from protein; (*b*) purified free from protein and phytin. † As in Table 1.
‡ Calc. by assuming $D_m = 0{\cdot}53 \times 10^{-7}$ for total polysaccharide and phytoglycogen A, and $D_m = 3{\cdot}5 \times 10^{-7}$ for phytoglycogen B.

TABLE 3. *Results of light-scattering measurements.*

Sample	Ref.	Uncorrected $M(\times 10^{-6})$	Dissymmetry	$1/(P_{90°})$	Corrected $M(\times 10^{-6})$
Phytoglycogen A	1	18	3·0	2·0	(21) *
,, B	1	4	1·18	1·12	4·5
		3·9	1·16	1·11	4·3
55—60% AcOH fraction ...	1	18	3·0	2·0	(21) *
Total polysaccharide	This work	22	1·26	1·17	26
		22	1·45	1·28	28

* Calc. by assuming a particle-scattering factor of 1·17.

found. Within experimental error, (Hc/τ) was independent of c for the range of concentrations studied, and hence the term $2B$[illegible]c/RT was negligible. The reproducibility of results for a given sample is indicated in Table 3.

Results.—The properties of the polysaccharide samples supplied by Professor Peat [1] are shown in Table 1. Table 2 shows the corresponding properties of our own samples. Table 3 summarizes the light-scattering data.

The results of typical sedimentation experiments are shown in the Figure.

DISCUSSION

Our lower yield of water-soluble polysaccharide (cf. ref. 1) is not thought to be due to degradation, since the amount of simple sugars was low, and the molecular weight of the product was high; it is more probable that the amount of this polysaccharide present in the seed varies.

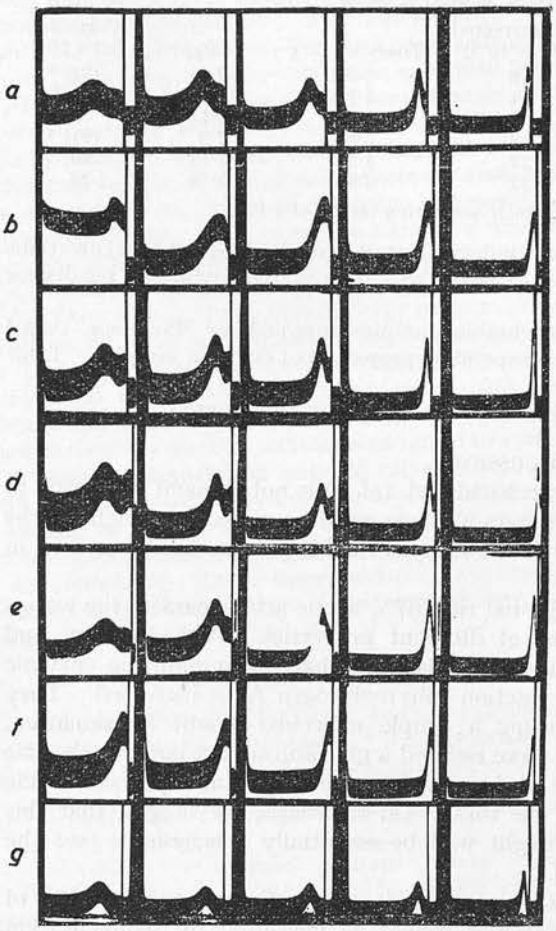
Previous workers (see ref. 1) have suggested that 67% acetic acid separates the water-soluble polysaccharide into two fractions of different properties. Peat, Whelan, and Turvey [1] have shown that these fractions are similar and that, by minimizing enzymic degradation, the percentage of insoluble fraction (phytoglycogen A) is increased. They concluded that the acetic acid was causing a simple molecular-weight fractionation. However, from the batch of seed used we have isolated a glucosan with a larger molecular weight than the phytoglycogen A of these workers, and our yield of soluble polysaccharide (phytoglycogen B) was less than 2% of the total. On this basis, we suggest that this sample of water-soluble polysaccharide might well be essentially *homogeneous* [see the sedimentation diagrams (*g*) in the Figure].

Molecular Weights of the Water-soluble Polysaccharide and its Fractions.—For most of the samples, the agreement between molecular weight as measured by sedimentation and diffusion and by light-scattering is good. This suggests that true molecular weights are being measured. The general conclusions drawn by Peat and his co-workers [1] regarding the drastic effect of enzymic degradation during isolation—unless precautions are taken to avoid this—are completely substantiated from a comparison of the molecular weights quoted in Table 1. These values are larger than the previous relative ones since the diffusion constant was smaller than estimated. Nearly all the samples are somewhat polydisperse on ultracentrifugation [see diagrams (*a*)—(*f*) in the Figure]. This suggests that material of low molecular weight may be present, or that some degradation had occurred during both isolation and subfractionation. The values for the subfractions

(Table 1) may suggest that a good separation of products of different molecular weight was not obtained.

The molecular weight of the total water-soluble polysaccharide from the sample of seed examined is about 30×10^6 (*i.e.*, $\overline{\text{D.P.}} \approx 180{,}000$). This value is larger than that reported for glycogens,[7] with the exception of the recent work by Stetten, Katzen, and Stetten.[8] The frictional ratio ($f/f_0 = 2$; calculated from the sedimentation and diffusion results) indicated that in solution the polysaccharide was heavily hydrated or was not spherical.

Typical sedimentation diagrams. All samples from Peat, Whelan, and Turvey[1] *[except (g)]. The concentration is* 1·0 g./100 ml. *except for (g) where it is* 0·5 g./100 ml. *Sedimentation is from right to left. The figures in parentheses after the times indicate the angle of the Schlieren bar.*



Phytoglycogen A: *speed* = 20,000 *r.p.m.*; 2(65°), 4·5(50°), 9·5(40°), 13·5(30°), *and* 18·5(30°) *min. after reaching full speed.*

Phytoglycogen B: *speed* = 20,000 *r.p.m.*; 2·5(70°), 6(60°), 10(45°), 16·5(30°), *and* 24(30°) *min. after reaching full speed.*

55—60% AcOH *fraction*: *speed* = 20,000 *r.p.m.*; 1(65°), 4(50°), 7(35°), 12(25°), *and* 17(25°) *min. after reaching full speed.*

60—65% AcOH *fraction*: *speed* = 20,000 *r.p.m.*; 2(65°), 5(50°), 10(40°), 14(30°), *and* 22(20°) *min. after reaching full speed.*

65—70% AcOH *fraction*: *speed* = 20,000 *r.p.m.*; 5(60°), 8·5(50°), 20(35°), 25(30°), *and* 36(30°) *min. after reaching full speed.*

>70% AcOH *fraction*: *speed* = 20,000 *r.p.m.*; 10(60°), 20(45°), 32(35°), 48(30°), *and* 65(25°) *min. after reaching full speed.*

Whole water-soluble polysaccharide: *speed* = 15,000 *r.p.m.* 5(70°), 9(60°), 12(55°), 15(55°), *and* 17(45°) *min. after reaching full speed.*

Structure of the Water-soluble Polysaccharide.—Our periodate oxidation results (chain-length = 14) are in general agreement with those of Peat and his co-workers.[1] These authors suggested that the polysaccharide must possess a glycogen-type structure. It is certainly true that the sedimentation behaviour is similar to that of glycogen rather than of amylopectin. (We have shown recently [9] that, whereas S_{20} is virtually independent of c for glycogens, for amylopectin S_{20} is very strongly dependent for c. This must be related to differences in shape of the two molecules.) However, a considerable difference occurs between the iodine-binding power of this type of polysaccharide and of glycogen. We suggested previously [6] that, since the uptake was 3—4 times greater than for a glycogen

of corresponding chain-length, the structure of the two polysaccharides differs in that the degree of multiple-branching of the water-soluble material is intermediate between those for glycogen and for amylopectin. However, in view of unit-chain-length, enzymic degradation experiments,[1] and sedimentation behaviour, the polysaccharide appears to be nearer in structure to a glycogen than an amylopectin.

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137. *Physicochemical Studies in Starches. Part XI.* The Granular Starch of Sweet Corn,* Zea mays.

By C. T. Greenwood and P. C. Das Gupta.

The starch present in sweet corn, *Z. mays*, has been isolated in granular form and obtained free from protein. It has been fractionated into its component amylose and amylopectin, and some physical properties of these fractions, including estimations of molecular weight, are described.

In view of recent interest [1] in the structure of the water-soluble glucosans of sweet corn (*Zea mays*), we have examined the *granular* starch which co-exists in the cereal grain. No detailed study of this starch appears to have been made previously.

Experimental

Isolation of Starch.—Mature *Zea mays* (var. Golden Bantam; 300 g.) was ground to a coarse powder (Found: H_2O, 12·3; ash, 1·81; N, 1·86%) and exhaustively extracted with boiling benzene–methanol (2 : 1 v/v; 2 l.) (Found: loss in wt., 12·3%). Defatted grain was then shaken vigorously with 0·1M-sodium chloride (1·5 l.; 5 × 6 hr.) under toluene, the aqueous suspensions filtered through muslin, and crude starch (A) allowed to settle (Found: N, 1·38%).

Removal of Protein from Starch A.—The crude starch was suspended in 1M-sodium chloride, then treated with toluene as previously described.[2] The process was repeated to yield starch B, which was stored in saline under toluene at 0° (yield, 5% of original dry wt. of grain) (Found: N, 0·13%).

Properties of Starch B.—On hydrolysis with 2% sulphuric acid, the starch yielded 96% of the theoretical amount of glucose (quantitative chromatography), and no other sugar was detected on the paper chromatogram. This material had $[\alpha]_D^{17}$ +152° (*c* 0·77% in N-NaOH). The optical density of the colour developed when starch (1 mg.) was stained with iodine (2 mg.) and potassium iodide (20 mg.) in distilled water (100 ml.) and measured at 6800 Å in cells of 2 cm. length (against the same iodine solution by means of a Unicam spectrophotometer) was 0·23. The iodine affinity of the starch (see below) was 5·5%, corresponding to 28% of amylose, whilst the average length of unit chain of the amylopectin was shown by calculation from the results of periodate oxidation [3] of the whole starch, and of the isolated amylopectin, to be 23 glucose residues.

Starch B was used in all further investigations.

Fractionation Methods.—Attempts were made to fractionate the *Z. mays* starch by the methods previously described in this Series: (i) dispersion in water at 98° followed by the addition of thymol as precipitant and butan-1-ol as the recrystallisation agent,[4] (ii) aqueous leaching at 70° followed by dispersion of the residue at 98° and addition of butan-1-ol,[4] and (iii) pretreatment of the granules with M-potassium hydroxide at 0°, before neutralization and dispersion and the addition of pentyl alcohol.[5, 6]

Characterisation of the Components.—These were characterized by (i) potentiometric titrations to determine iodine affinity,[7] (ii) measurements of limiting viscosity number $[\eta]$ in M-potassium hydroxide,[8] and (iii) measurements of sedimentation velocity in 0·2M-potassium hydroxide.

Measurement of Sedimentation Velocity.—Rates of sedimentation of the two components in 0·2M-potassium hydroxide were determined by using a " Spinco " electrically driven ultracentrifuge (Spinco Division, Beckman Instruments Corporation, Belmont, California). Measurements were made in a 12 mm. cell incorporating a Kel-F centrepiece. The initial experiments with alkali as a solvent for the components [9] showed that the optimum speed for solutions of amylose of concentration greater than 0·1 g./100 ml. was 60,000 r.p.m., whilst for more dilute solutions, 30,000 r.p.m. was more suitable. Amylopectin solutions were spun at either 15,000 r.p.m. or 12,600 r.p.m. depending on the concentration. The pressure in the vacuum-chamber was less than 1 μ Hg, and the temperature rise in the rotor was about 0·6°/hr. at 60,000 r.p.m. and correspondingly less for lower speeds. (The refrigerating system was not

* Part X, preceding paper.

utilized.) Runs were normally complete within 30 min. The temperature of the rotor was measured before and after the completion of each run, and the temperature at any time during the run obtained by linear interpolation. A correction was applied to allow for the adiabatic cooling of the rotor during acceleration.[10, 11] A modified Philpot–Svensson optical system enabled movement of the boundaries to be followed directly. The magnification of this lens system was shown to be constant over the whole field. The distance from the reference line of the optical system to the axis of rotation was 5·730 cm. Measurements of the rates of sedimentation were made by measuring the position of the boundary to 0·01 mm. directly from the photographic plates, a two-dimensional travelling microscope being used. Sedimentation constants (S) were evaluated from the equation: $\mathrm{d}(\log_{10} X/\omega^2)\mathrm{d}t = S/2{\cdot}303$, X being in cm. from the centre of rotation, t the time from the start of the acceleration in sec., and ω the angular velocity in radians. In this manner, linear graphs of $\log_{10} X$ against t were obtained: the point where the lines cut the time-axis at the value of $\log_{10} X$ for the meniscus, representing the time required before sedimentation started (*i.e.*, approx. two-thirds of the acceleration period). Results were corrected [12] to water at 20°.

Diffusion Measurements.—The method is outlined in Part X.[13] The solvent was 0·2M-potassium hydroxide, and values of the diffusion constant (D_m) were calculated by the moment method.

Results and Discussion

Zea mays starch has been isolated in granular form, reagents likely to cause degradation being avoided. Contamination with protein was reduced to 0·8% under the necessary mild conditions by the technique previously described.[2] However, purely physical methods did not further reduce the protein content. This phenomenon has been encountered [14] during the isolation of a large number of starches. The significance of the protein is doubtful, although it may well be incorporated into the granular structure.

The starch proved difficult to disperse in hot water. This is a feature which has been found in this laboratory to be common to a large number of cereal starches. Its significance with regard to granular structure is not yet known. The apparent resistance of the granule made its fractionation difficult. Conventional aqueous dispersive and leaching methods of fractionation were, in fact, unsuccessful, and the method involving preliminary swelling in M-potassium hydroxide [5, 6] had to be used. The properties of the components from the most effective separation achieved by this method were as shown in the Table.

Properties of Z. mays *starch components.*

	Iodine affinity [a]	Purity (%)	$[\eta]$ in M-KOH	$10^{13}(S_{20})_0$ [b]	$\overline{M}_{S,D}$ [c]	$\overline{M}_n$
Amylose	18·8 *	100	150	5·2	317,000	180,000
Amylopectin	0·3	98	100	275	—	—

[a] See ref. 7. [b] Values obtained by graphical extrapolation, see text. [c] Calc. from $\overline{M}_{S,D} = \boldsymbol{R}T(S_{20})_0/(1 - \overline{V}\rho)D_m$.
* Constant on further recrystallization.

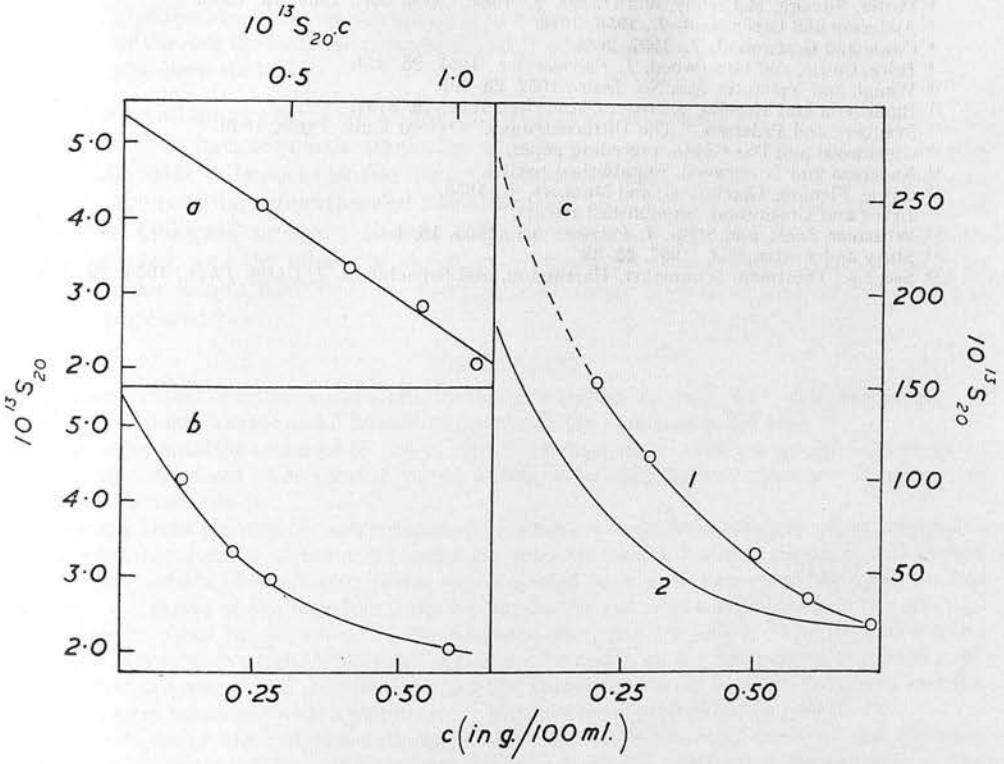
The iodine affinity of the amylose is slightly lower than that for potato amylose, but similar to that found for other cereal starches in this laboratory. Barriers to β-amylolysis are present. Enzymic experiments carried out by Mr. A. W. Arbuckle showed that treatment with pure β-amylase resulted in only 78% conversion into maltose, a value very similar to the 77% limit reported for potato amylose.[15] The concurrent action of β-amylase and Z-enzyme resulted in 100% conversion, thus giving a further check that the amount of amylopectin impurity was negligible.

As reported previously,[9] the sedimentation constant (S_{20}) for both components is strongly dependent on the concentration c (see Figure). For amylose, the extrapolation to infinite dilution was simplified by plotting S_{20} against $S_{20}.c$ as well as against c. The resultant value $(S_{20})_0$ was $5{\cdot}2 \times 10^{-13}$ c.g.s. units. The diffusion coefficient (D_m) at $c = 0{\cdot}2$ g./100 ml. was measured by Mr. W. A. J. Bryce who found $D_m = 1{\cdot}0 \times 10^{-7}$, and hence, when the partial specific volume ($\overline{V}$) of amylose in this solvent [16] is taken to be

0·60, the molecular weight of the amylose ($\overline{M}_{S,D}$) is 317,000 and its D.P. = 1950. Calculation of the number-average molecular weight ($\overline{M}_n$) from the viscosity data by the relation previously obtained for potato amylose [4] gave $\overline{M}_n$ 180,000 and D.P. 1100. These values are lower than those for potato amylose.

Whilst the sedimentation diagrams for the amylose were well defined at low dilutions, those for the amylopectin showed a great tendency to spread and so make measurement of the mean position of the peak difficult. In view of the extreme concentration

The variation of sedimentation constant (S_{20}) with concentration (c) for (a) amylose, S_{20} versus $S_{20}.c$, (b) amylose, S_{20} versus c, (c) amylopectin, S_{20} versus c for (1) Z. mays and (2) potato.



dependence, no attempt was made to extrapolate S_{20} accurately to infinite dilution, but for comparison our previous results [9] for potato amylopectin are also shown in the Figure. Although without knowledge of the diffusion coefficient the molecular weight cannot be estimated, this would appear to be greater than for potato amylopectin for which values of 36×10^6 have been found.[17] This extreme size might well cause the difficulties in dispersion and fractionation of the starch. Ultracentrifugal examination of some of the more impure amylopectins showed two peaks, in agreement with Stacy and Foster's recent results.[18] However, any appreciable amylose impurity (greater than about 5%) is sufficient to distort completely the sedimentation diagram at low concentrations of amylopectin. This phenomenon, together with boundary anomaly effects,[19] makes reliable estimates of S_{20} and its consequent extrapolation to infinite dilution impossible. Experience in this laboratory has shown that ultracentrifugal analysis of impure amylopectins is most unsatisfactory (cf. ref. 18).

The granular starch in *Z. mays* is obviously a typical cereal starch, and its properties are completely unaltered by the co-existence in the cereal grain of the glycogen-like water-soluble glucosans.[1,13]

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138. *Physicochemical Studies on Starches. Part XII.* The Molecular Weight of Glycogens in Aqueous Solution.*

By W. A. J. Bryce, C. T. Greenwood, I. G. Jones, and D. J. Manners.

Molecular weights are presented for 23 samples of glycogens isolated from various biological sources. Ultracentrifugal analysis showed that most of the samples were polydisperse. The molecular weights of the main components lie in the range (3—9) $\times 10^6$. The polydisperse nature of the glycogens has been confirmed by light-scattering measurements. The effects of varying the isolation procedure, and of alkali, on the molecular weight have also been studied.

Glycogen and amylopectin are both highly branched, essentially α-1 : 4-linked glucosans. However, their hydrodynamic properties are completely different. This must be related to fundamental differences in fine structure and molecular shape.[1,2] In this paper, we describe the solution properties and hydrodynamic behaviour of glycogens isolated from a variety of biological sources. Estimations of molecular weight and its distribution have been obtained, and the effects of variations in the method of isolation, and of alkali, on the molecular weight have been studied. A preliminary account of some of this work has already appeared.[3]

Experimental

Sedimentation Measurements.—The methods described in Part XI [4] were employed. M- and 0·1M-sodium chloride and 0·2M-potassium hydroxide were used as solvents.

The sedimentation constant (S_{20}) was virtually independent of the solvent, and the majority of the measurements were carried out in either M- or 0·1M-sodium chloride. Results were corrected to water at 20°.

The apparent amount of each component in a resolvable polydisperse system was estimated by direct measurement of the areas under the refractive-index gradient curves. An enlarged image (3×) of the photographic plates was projected on smooth paper and the upper outline traced. An image of the base line (from a comparative run with solvent alone in the cell) was then superimposed by alignment of the reference lines, and traced on. The refractive-index gradient curves were carefully divided, in the usual manner, on the assumption that each component had a symmetrical distribution, and the appropriate areas between the peaks and the base-line were measured with a planimeter. Values were expressed to the nearest 5%.

Estimations of the polymolecularity of the major component of some of the glycogen samples were obtained by using Gralen's function,[5] dB/dX, where B is an estimate of the "width" of the sedimentation gradient curve and is equal to A/H (A = area of the Schlieren diagram; H = the height of the maximum ordinate), and X = the distance of the peak from the axis of rotation. In all instances, B varied linearly with X. Although this function should be extrapolated to infinite dilution, the value at c = 1 g. per 100 ml. was taken as a standard for comparison of the polymolecularity of different samples.

Diffusion Measurements.—The method is outlined in Part X.[6] The solvent was 0·1M-sodium chloride, and values of the diffusion constant (D_m) were calculated by the moment method.

Partial Specific Volume.—The partial specific volume ($\bar{V}$) of glycogen was taken as 0·62, the value calculated from density measurements on aqueous solutions of one sample.

Light-scattering Measurements.—The apparatus and the methods used to clarify and dilute the glycogen solutions were similar to those previously described for the *Zea mays* polysaccharides,[6] 0·1M-sodium chloride being the solvent. Although 15% aqueous magnesium chloride has been recommended,[7,8] we found this solvent to have no advantages. The value of the molecular weight of a given sample was the same in both the above solvents. Glycogen

* Part XI, preceding paper.

solutions were clarified by careful filtration (cf. ref. 8) through sintered glass (G4). Repeated filtration caused some small loss in turbidity, whilst little improvement occurred in the apparent dissymmetry. (For example, a sample after one filtration had $M = 8{\cdot}4 \times 10^6$, dissymmetry = 1·41; after five filtrations, $M = 7{\cdot}7 \times 10^6$, dissymmetry = 1·34, the concentration being assumed to be unchanged by filtration.) Solutions were therefore filtered once, before dilution. This procedure gave reproducible results. Hc/τ was independent of c for all samples. The particle scattering factor ($P_{90°}$) was calculated from the dissymmetry, the molecules being assumed to be spherical.[6] The refractive-index increment (dn/dc) for glycogen was found to be 0·146 (c = g./ml.) in 0·1M-sodium chloride at 546 mμ.

Isolation of Glycogens.—Unless otherwise stated, samples of glycogen had been isolated from the tissue by the classical Pflüger method involving digestion with 30% aqueous potassium hydroxide at 100°, followed by precipitation of the glycogen with ethanol and with acetic acid.[9] Commercial samples of glycogen from British Drug Houses Ltd. (I), and Nutritional Biochemicals Corporation, Ohio, U.S.A. (II), were also examined. Methylated horse-muscle glycogen was kindly provided by Dr. D. J. Bell.

Results and Discussion

Sedimentation Coefficients.—Typical sedimentation data are shown in Table 1. It was apparent that for all the glycogens studied in detail, the sedimentation constant (S_{20}) was dependent on the concentration (c), and varied by about 10% for a 1% change in concentration. This is in general agreement with Larner, Ray, and Crandall's results,[10] but, whilst these authors suggested that S_{20} was a function of c^2, our values were best

Table 1. *Typical sedimentation results.*

		$10^{13}S_{20}$ at c (g./100 ml.)							
Glycogen sample	Solvent	1·0	0·75	0·50	0·25	0·16	0·125	0·08	0 (extrapol.)
Ascaris lumbricoides ...	0·1M-NaCl	47	47	47	48	—	—	—	48
Brewer's yeast	0·1M-NaCl	56	—	60	61	—	62	—	64
,, ...	1M-NaCl	54	—	—	—	—	—	—	—
,, ...	0·2M-NaOH	56	58	60	61	62	—	63	64
Commercial, II	0·1M-NaCl	65	67	69	71	—	—	—	73

represented by a linear function. The relations was expressed by $S_{20} = (S_{20})_0(1 - kc)$, where $(S_{20})_0$ is the value of S_{20} at infinite dilution, and c was expressed in g./100 ml. With the exception of the *Ascaris lumbricoides* glycogen (which was relatively concentration-independent; see Table 1), the average value of k was $0{\cdot}10 \pm 0{\cdot}02$. Values of $(S_{20})_0$ for glycogens examined at only one concentration were therefore calculated from this value, and are shown in parentheses in the second and third columns of Table 2.

Molecular Weight and its Distribution.—Table 2 shows the results of the sedimentation measurements for the 23 samples examined. Typical sedimentation diagrams are shown in the Figure. Most samples proved to be polydisperse on ultracentrifugation. Diagrams *a* and *b* (for oyster and *Helix pomatia* glycogen) illustrate the type of Schlieren diagram observed for the most obviously polydisperse samples. This feature is unusual, although Polglase, Brown, and Smith [11] reported similar results for samples of human-liver glycogen. The amounts of main components quoted in the Table are only approximate as no attempt was made to correct for boundary anomaly effects.[12] For many samples, an extremely wide molecular-weight distribution was indicated; the leading sedimentation boundary was extremely asymmetric and reached nearly to the bottom of the cell after a short time of centrifugation. It was difficult to prove whether or not this leading boundary was a second component, and hence no attempt was made to estimate either its amount or its approximate sedimentation constant. Samples which showed this probable fast component are indicated by the symbol S_{20}(F) in the Table. In some other samples, a corresponding asymmetric lower molecular weight distribution was apparent. Again, no analysis of this was attempted and this is indicated by the symbol S_{20}(S) in Table 2.

Diffusion measurements showed that for methylated horse muscle the diffusion coefficient (D_m) = $1{\cdot}0 \times 10^{-7}$; for brewer's yeast glycogen, $D_m = 1{\cdot}1 \times 10^{-7}$; for com-

mercial glycogen I, $D_m = 2{\cdot}0 \times 10^{-7}$; and for commercial glycogen II, $D_m = 1{\cdot}1 \times 10^{-7}$ The molecular weights shown in Table 2 for the main components in the other samples are calculated by assuming a value of $1{\cdot}1 \times 10^{-7}$ for D_m in agreement with other workers.[2] All the molecular weights are in the range (3—9) $\times 10^6$, and, together with the values of the frictional ratio (f/f_0), are of the same order as those previously reported from sedimentation and diffusion measurements.[2,13] It should be noted, however, that the values for

Typical sedimentation diagrams. For all samples, c = 1 g./100 ml.; solvent, 1·0M-sodium chloride; speed = 20,000 r.p.m. Movement of the peaks is from right to left. The figures in parentheses after the times indicate the angle of the Schlieren bar.

Oyster glycogen: 8 (55°), 15 (45°), 18 (35°), 30 (30°), *and* 47 (25°) *min. after reaching full speed.*

Helix pomatia *glycogen*: 6 (65°), 14 (45°), 25 (45°) 37 (45°), *and* 50 (35°) *min. after reaching full speed.*

Brewer's yeast glycogen: 9 (65°), 17 (50°), 26 (45°), 37 (40°), *and* 52 (40°) *min. after reaching full speed.*

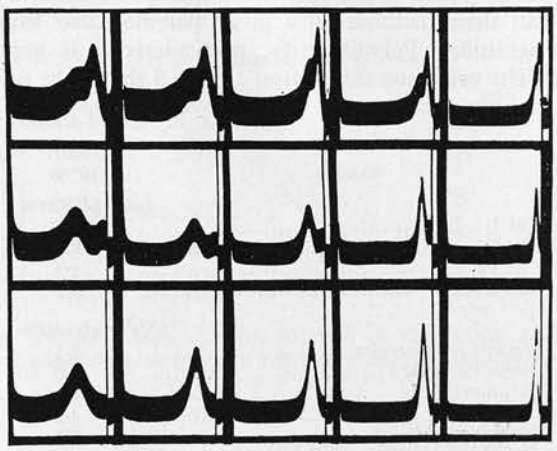

TABLE 2. *Sedimentation results.*

Glycogen sample	$10^{13}S_{20}$ of components [a] major	minor	Major component (%)	dB/dx [b]	$10^{-6}M$ [c]	f/f_0 [d]
	(a) *Mammalian livers*					
Cat I	75	F, S	—	0·8	4·4	—
,, IV	84	F, S	—	—	4·9	—
,, VI	(102)	F, S	—	1·0	5·9	—
Human (glycogen-storage disease)	(53)	(220)	70	1·8	3·1	—
Fœtal sheep	110	F	—	1·1	6·4	—
Fœtal pig	(49)	(11)	70	0·8	2·9	—
Rabbit II	94	—	95+	—	5·5	1·7
,, (fructose-infused *)	(80)	F	—	1·1	4·7	—
,, (galactose-infused *)	(153)	S	—	—	9·0	—
,, (normal *)	(145)	F	—	1·8	8·4	—
	(b) *Mammalian muscles*					
Horse (methylated)	23	—	95+	—	2·8	1·4
Human	(85)	(20)	85	0·8	4·9	—
Rabbit I	79	—	95+	0·7	4·6	1·9
	(c) *Other glycogens*					
Ascaris lumbricoides	48	F	—	—	2·8	—
Brewer's yeast	64	—	95+	—	3·7	2·0
Commercial, I	24	—	95+	—	0·7	1·9
,, II	73	—	95+	—	4·0	1·7
Helix pomatia II	(63)	(300, 7)	80	0·9	3·6	—
Mytilus edulis I	(93)	F	—	0·9	5·4	—
Oyster *	(45)	(90, F)	—	—	2·6	—
Tetrahymena pyriformis I	(69)	S	—	—	4·0	—
Trichomonas foetus I	(70)	S	—	—	4·0	—
Trichomonas gallinae I	(84)	S	—	—	4·9	—

[a] For values in parentheses and meaning of F and S, see text. [b] Value for main component at c (total) = 1·00 g./100 ml. [c] Molecular weight calculated from $M = \boldsymbol{R}T(S_{20})_0/(1 - \overline{V}\rho)D_m$. [d] Frictional ratio calculated from $f/f_0 = 10^{-8}[(1 - \overline{V}\rho)/D_m{}^2(S_{20})_0\overline{V}]^{1/3}$.

* Samples kindly supplied by Dr. M. Schlamowitz.

rabbit liver and muscle are considerably lower than those recently reported by Stetten, Katzen, and Stetten [8] (see below).

The values of (dB/dx) confirm qualitatively the extremely polymolecular nature of glycogen (cf. ref. 5) in agreement with the distributions evaluated by Larner and his co-workers.[10] Further, in agreement with these authors, mammalian-muscle glycogens appear to be less polymolecular than liver glycogens.

The polydisperse nature of most of the samples studied was confirmed by turbidimetric measurements. Although the molecular weight from these measurements is a true weight-average whilst that from sedimentation and diffusion is less well-defined,[14] the results from both these methods on a given polymolecular sample should be of the same order of magnitude. Polydispersity, particularly if it involves components of high molecular weight will cause disparities. Table 3 shows the results of light-scattering measurements

TABLE 3. *Light-scattering results.*

Sample	Uncorr. 10^{-6}M	Dissymmetry	$1/P_{90°}$	Corr. 10^{-6}M
Liver glycogens				
Cat I	10·5	1·48	1·30	13·6
,, IV	8·8	1·85	1·52	13·4
,, VI	12·8	1·67	1·40	17·9
Rabbit II *	6·9	1·20	1·13	7·8
Other glycogens				
Ascaris lumbricoides	7·1	1·40	1·26	8·9
Brewer's yeast *	4·0	1·15	1·10	4·4
Commercial, I	1·7	1·19	1·12	1·9
,, II *	4·9	1·15	1·10	5·4
Rabbit muscle I *	3·7	1·17	1·11	4·1
Tetrahymena pyriformis I	6·1	2·50	1·85	11·3

* Samples exhibiting no polydispersity.

on the ten samples which appeared to be the least obviously polydisperse on ultracentrifugation. For four of these, the agreement is reasonably good, indicating that they were only polymolecular, whilst the presence of S_{20}(F) in the other samples is convincingly illustrated by the higher turbidimetric molecular weight. It is therefore suggested that a given glycogen sample should be examined by both the sedimentation and the light-scattering method in order to prove unambiguously whether or not it is polydisperse. Without further investigations, it is not possible to decide whether polydispersity occurs in native glycogen in the tissue or is an artefact resulting from degradation during isolation. Polglase and his co-workers [11] consider that such variations occur naturally.

Effect of Isolation Procedure on Molecular Weight.—The classical Pflüger method involving digestion of tissue with 30% potassium hydroxide has often been criticised [15] on the assumption that alkaline degradation occurs. Table 4 shows the results for the determination of S_{20} for glycogen samples isolated from the halves of two rabbit livers severally with boiling water and 30% aqueous potassium hydroxide. Within experimental error, S_{20} is the same for all samples. It is concluded that, in the presence of air, the extent of degradation of glycogen by 30% potassium hydroxide solution at 100° is no greater than that which might be caused by boiling water. Similar results have been obtained by Staudinger,[16] and Bridgman [17] reported that glycogen extracted with cold trichloroacetic acid and hot alkali from two halves of a rabbit liver had a similar molecular weight. However, recent light-scattering work by Stetten, Katzen, and Stetten [8] has shown that if extraction with trichloroacetic acid is for a limited time at 0° the glycogen from rabbit liver has an average molecular weight of $(11—80) \times 10^6$ rather than the $(2—6) \times 10^6$ as in hot potassium hydroxide extractions. This suggests that it is difficult to avoid degradation during extraction, and that the molecular weights reported here and previously [2] may not be representative of " native " glycogen.

Effect of Dilute Alkali and Acetic Acid.—In contrast to the behaviour of hot 30% alkali,

hot *dilute* alkali appears to degrade glycogen rapidly. Digestion of rabbit-liver glycogen in 8% aqueous sodium hydroxide at 100° for 1·5 hr. reduced S_{20} from 86 to 57 $\times$ 10^{-13} c.g.s. units (see Table 4), and increased the polymolecularity (as shown by a broadening of the peak of the Schlieren pattern).

It has been suggested [18] that purification of glycogen by precipitation with glacial acetic acid may render it unsuitable for ultracentrifugal analysis. However, when rabbit-liver and brewer's yeast glycogens were reprecipitated with 80% acetic acid there was no change in the value of S_{20} (see Table 4). Precipitation of glycogen by acetic acid does not, therefore, alter the hydrodynamic properties or cause degradation of glucosidic linkages to any appreciable extent.

TABLE 4. *Effect of isolation procedure on the sedimentation constant.*

Sample	Method of isolation	$10^{13}S_{20}$ at c = 1 g./100 ml.
Rabbit liver XII	Hot water	85
	30% KOH at 100°	86
Rabbit liver XIII	Hot water	76
	30% KOH	83
	30% KOH + repptn. with AcOH	83
Rabbit liver IV	30% KOH	86
	30% KOH + 8% NaOH at 100° for 1½ hr.	57
Brewer's yeast	30% KOH	64
	30% KOH + repptn. with AcOH	63

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535. *Physicochemical Studies on Starches. Part XIII.* The Fractionation of Oat and Wheat Starches.*

By A. W. Arbuckle and C. T. Greenwood.

The behaviour of laboratory-prepared oat and wheat starches on fractionation by dispersion and aqueous leaching has been critically examined. Aqueous leaching at various temperatures resulted in sub-fractionation of the amylose. The physical properties of the various starch-products have been determined and are discussed.

Satisfactory fractionation of laboratory-prepared cereal starches into their component amylose and amylopectin appears to be more difficult [1,2] than is the case for potato starch. This paper deals with the problems for oat and wheat. The effect of variations in methods of fractionation on molecular size and β-amylolysis limits of the amylose has been examined. Results are discussed with regard to the structure of amylose and the nature of the granule.

Experimental Methods

Preparation of the Starches.—Starch was isolated from oats (var. Milford) and wheat (var. Victor II) by aqueous extraction of the defatted grain, and was purified from protein by Anderson and Greenwood's method.[3] The starches were then exhaustively defatted with boiling 80% methanol. A commercial sample of wheat starch was also studied.

Leaching and Dispersing Procedures.—The methods employed to fractionate the starches in a nitrogen atmosphere by (i) aqueous leaching at 70° and 98°, and (ii) complete dispersion of the granular structure with and without pretreatment with M-potassium hydroxide at 0° were as described elsewhere,[4] with the exception that some dispersions were carried out in the presence of phosphate buffer (pH 6·5; 2 ml./100 ml. of dispersion).

Characterization of Starch Products.—Measurements were made of (i) iodine affinity, (ii) limiting viscosity number $[\eta]$ in M-potassium hydroxide, and (iii) β-amylolysis conversion limit. (See earlier papers in this Series.)

Properties of Whole Starches.—The properties of the whole starches were:

Starch	Protein (%) [a]	I.A.[b]	Amylose (%) [c]	$\overline{R}$ [d]	C.L. for amylopectin [e]
Oat	0·24	5·13	27·0	26·8	19·6
Wheat	0·33	5·00	26·3	25·6	18·9

[a] % N × 6·25. [b] Iodine affinity. [c] For both starches (I.A./19·0) × 100. [d] Ratio of terminal to non-terminal groups from HIO_4 oxidation (see ref. 5). [e] Conversion limit, calc. from previous column.

Results and Discussion

For both starches, the purely physical methods of purification used did not reduce contaminating protein to below about 0·3%, a value about ten times greater than that for potato starch. Johnston's method [6] involving extraction with 1% ammonium oxalate was also not satisfactory. Tightly bound protein appears to be, in fact, a characteristic of these laboratory-prepared granular starches. Its significance with regard to granular structure is not known, but it might well hinder the swelling properties and so influence its dispersive properties. The salient features of the results of various fractionation

* Part XII, *J.*, 1958, 711.

experiments are summarized in Tables 1 and 2. Values for the average degree of polymerization of the amylose ($\overline{D.P.}$) were calculated by using the relation previously obtained for potato amylose.[4] Although this may not be extremely accurate, the calculated values are likely to be of the correct order of magnitude.

Whilst sub-fractionation of the amylose component by aqueous leaching of the granules at various temperatures was satisfactory, both starches proved extremely difficult to disperse before conventional fractionation by precipitants. Resulting dispersions were turbid even after two hours' boiling, and addition of either sodium chloride or phosphate buffer (pH 6·47) made no improvement. Preliminary experiments with oat starch made in collaboration with Mr. J. M. G. Cowie had shown thymol to be a more

TABLE 1. *Properties of the components from oat starch.*

		Amylopectin		Amylose				
Expt.	Method of fractionation	Purity (%) [a]	% of total amylose retained [b]	I.A.[c]	$[\eta]$ in M-KOH	D.P.[d]	Conversion limit [e] (i)	(ii)
F1	70° Aqueous leach	82	67	19·1	160	1190	95	100
F2	98° Aqueous leach	94	23	18·0	340	2500	79	97
F3	Thymol–Bu^{n}OH dispersion without buffer	92	31	13·7	180	1330	71	86
F4	Thymol–Bu^{n}OH dispersion with buffer (pH 6·47)	93	27	14·7	212	1570	—	—
F5	KOH-pretreatment	99	<4	16·8	263	1950	72	91

[a] Calc. from (iodine affinity/19·0) × 100. [b] % of total amylose in starch retained as impurity in the amylopectin. Calc. by assuming original starch to contain 27% of amylose. [c] Iodine affinity. [d] Approx. degree of polymerization. Calc. from $\overline{D.P.} = 7{\cdot}4\,[\eta]$ (see text). [e] β-Amylolysis limits for (i) pure β-amylase, and (ii) β-amylase + Z-enzyme. Expressed as % conversion into maltose. Accuracy ± 2%.

TABLE 2. *Properties of the components from wheat starch.*

		Amylopectin		Amylose				
Expt.	Method of fractionation	Purity (%) [a]	% of total amylose retained [b]	I.A.[c]	$[\eta]$ in M-KOH	D.P.[c]	Conversion limit [c] (i)	(ii)
F6	70° aqueous leach	81	74	17·8	145	1070	98	—
F7	98° aqueous leach	94	23	15·8	300	2240	66	89
F8	Thymol–Bu^{n}OH dispersion without buffer	96	12	19·0	260	1920	65	96
F9 *	Thymol–Bu^{n}OH dispersion without buffer	95	18	17·2	133	980	—	—
F10	Thymol–Bu^{n}OH dispersion with buffer (pH 6·47)	96	12	17·6	280	2060	—	—
F11	KOH-pretreatment	96	12	18·0	258	1910	—	—

[a] Calc. from (iodine affinity/19·0) × 100. [b] Calc. as for Table 1 by assuming original starch to contain 26·3% of amylose. [c] As for Table 1. * Commercial starch.

suitable initial precipitant than *cyclo*hexanol, butan-1-ol, pentanol, or pyridine. This reagent was used therefore for wheat. Difficulty was found in recrystallizing both amyloses from butan-1-ol solutions; in some instances, an iodine affinity of 19·0% (the maximum value found for these amyloses: cf. ref. 5) could not be achieved.

The limiting viscosity numbers of the 98°-leached products (F2 and F7) were higher than for the other amylose fractions. This suggests that degradation must have occurred during the dispersion—even in a nitrogen atmosphere—and the two amyloses appear to be more susceptible to hydrolysis than potato amylose. (For example, when amylose F2 was heated in boiling water in the presence of nitrogen for 1 hr., $[\eta]$ decreased from 340 to 260, whilst for amylose F7 under similar conditions $[\eta]$ decreased from 300 to 280.)

Fractionation Conditions.—In view of the apparent lability of the amylose components of oat and wheat starch, fractionation by aqueous leaching at 98° (cf. ref. 7) yields amylose of higher purity and limiting viscosity number than does a conventional dispersion. However, the purity of the amylopectin obtained by this method may not be high (cf. also refs.

4 and 8). For laboratory-prepared cereal starches, as has already been found for *Zea mays* starch,[2] the method [9] involving pretreatment with M-potassium hydroxide at 0° appears to be most satisfactory; it yields purer amylopectin, and amylose which is relatively little degraded.

Fractionation of Commercial Wheat Starch.—The sample of commercial wheat starch gave an amylose with a low limiting viscosity number. The decrease was relatively much larger than between laboratory- and commercially-prepared potato starch.[4] This is related perhaps to the more vigorous purification necessary to remove protein in the manufacture of cereal starches.

Uniformity of Structure of Amylose.—To investigate whether the different amylose fractions were linear or contained some branch-point or other anomaly, β-amylolysis experiments were carried out (see Tables 1 and 2). The concurrent action of β-amylase and Z-enzyme [10] enabled another calculation to be made of the amount of amylopectin impurity in some fractions; this value agreed well with that from iodine affinity measurements. Table 3 summarises the results and previous values obtained for potato amylose.[4]

TABLE 3. *Comparison of β-amylolysis limits for various fractions of amylose from laboratory-prepared starches.*

	Potato †			Oat			Wheat		
Prep. of sample in N_2	% of total amylose	$[\eta]$	β-limit *	% of total amylose	$[\eta]$	β-limit *	% of total amylose	$[\eta]$	β-limit *
Aq. leaching at 70°	40	240	100	33	160	95	28	145	98
Aq. leaching at 98°	80	370	86	77	340	80	77	300	69
Dispersion of granule ...	100	440	77	100	180	77	100	260	66

† Results from ref. 4. * Expressed as % conversion into maltose. Calculated by assuming 56% conversion of any amylopectin impurity into maltose.

The same general trend for the β-amylolysis limits of the different fractions is observed as for potato starch.[4] Leaching at 70° caused limited swelling of the granules and enabled short-chain, essentially linear amylose (as shown by its high conversion into maltose) to diffuse out. Extraction at higher temperatures gave amylose which was incompletely hydrolysed, the amount of resistant material increasing with increase in temperature and consequent swelling and disruption of the granule. The difficulties in determining the nature of the barrier to β-amylolysis have been discussed elsewhere,[11] but it is thought that since disruption of the granule is involved, branching in the amylose is not improbable.[4]

For oat amylose, if about 35% is linear, and the whole is hydrolysed to 77% with β-amylase, then the portion containing an anomaly must be hydrolysed to about 65%. For wheat amylose, similar calculations show that the portion containing an anomaly must be hydrolysed to about 50%. These results suggest that the barrier to β-amylolysis in these amyloses is again essentially randomly situated (cf. ref. 10).

Although Peat,[12] Hopkins,[13] and Hassid,[14] with their collaborators, have suggested that pure β-amylase converts only about 70% of amylose into maltose, our experiments on amylose sub-fractions indicate that amyloses obtained by dispersive methods consist of two types of molecule, some being linear and others having a randomly situated barrier to this enzymic hydrolysis. On this basis, the percentage of linear material (L) in any amylose fraction can be calculated to the first approximation from the expression: $(T - L)/(100 - L) = 1/2$, where T = the percentage conversion into maltose for the total fraction. Values of L from calculation and experimental determination may not agree, since the aqueous leaching is not necessarily quantitative. The results from such calculations (*i.e.*, potato, *ca.* 55; oat, *ca.* 55; wheat, *ca.* 30%) suggest that the amount of linear material may vary from starch to starch.

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536. *Physiochemical Studies on Starches. Part XIV.*[1] *The Effect of Acid on Wheat-starch Granules.*

By A. W. Arbuckle and C. T. Greenwood.

The effect of acid on potato-starch granules has been described in Part V.[2] During further studies of granular structure, its effect on wheat-starch granules has been investigated.

Experimental.—Wheat starch (var. Victor II) was isolated and purified as in Part XIII.[1] Methods used to treat the granules with acid in a nitrogen atmosphere, to measure the overall effect of acid-treatment on the granules, and to characterize the fractionation products were as for the previous investigation on potato starch.[2]

Initial experiments were carried out by treating the granules with 0·2M-hydrochloric acid at 45°. Potentiometric iodine titration on the products showed changes analogous to those observed for potato granules: there was an apparent increase in iodine-uptake for the first 2 hr., and a decrease thereafter. In addition, the slope of the linear portion of the curve increased with time of acid-treatment, as did the activity of free iodine necessary to saturate the amylose. These results and those obtained on treating the granules with M-hydrochloric acid at 16° were as shown.

Time of acid-treatment (hr.)		0	1	2	4	6	8	24
For 0·2M-HCl at 45°	Iodine affinity	5·0	5·2	5·5	4·4	3·8	—	1·5
	Amylose (%) †	26·3	27·3	29·0	23·7	20·0	—	7·9
For M-HCl at 16°	Iodine affinity	5·0	—	5·3	5·7	—	5·6	4·8
	Amylose (%) †	26·3	—	28·0	30·0	—	29·5	25·3

† Apparent % of amylose calc. by assuming iodine affinity of amylose = 19·0 (see ref. 5).

The properties of the fractionated components from the acid-treated starches were as shown in Table 1. Degrees of polymerization ($\overline{D.P.}$) were calculated on the assumption that the relation found for potato amylose [3] holds in order to show the order of magnitude of the changes involved. Fractionation of the acid-treated starches proved more difficult than for the original starch. A similar effect was found by Kerr [4] for maize starch.

Table 1. *Properties of the fractionated components.*

0·2M-HCl at 45°

Acid-treatment (hr.)	Amylose			Amylopectin			
	I.A.[a]	$[\eta]$ [b]	$\overline{D.P.}$[c]	I.A.[a]	% Amylose [d]	$[\eta]$ [b]	$10^{13} S_{20}$ [e]
0	19·0	260	1920	0·5	3	140	121
1	10·3	178	1320	—	—	80	28
2	11·2	164	1210	—	—	60	22
4	12·5	140	1030	0·5	3	—	—
6	14·8	134	990	0·8	4	50	19
24	—	—	—	—	—	30	5

M-HCl at 16°

Acid-treatment (hr.)	Amylose			Amylopectin			
	I.A.[a]	$[\eta]$ [b]	$\overline{D.P.}$[c]	I.A.[a]	% Amylose [d]	$[\eta]$ [b]	$10^{13} S_{20}$ [e]
0	19·0	260	1920	0·5	3	140	121
2	12·6	235	1740	0·7	4	175	110
4	16·8	212	1570	1·5	8	110	80
8	18·7	188	1390	1·1	6	140	85
24	—	150	1110	0·8	4	160	56

[a] Iodine affinity (see ref. 5). [b] Measured in M-KOH. [c] Calc. from $\overline{D.P.} = 7{\cdot}4[\eta]$ (see ref. 3). [d] % of amylose impurity calc as in ref. 3. [e] Sedimentation constant in c.g.s. units at $c = 0{\cdot}2$ g./100 ml.

Discussion.—Potentiometric iodine titrations suggested that amylopectin was preferentially solubilized to give an increase in the apparent amount of linear material in the granule. The amount of reducing sugar in the acid supernatant liquid was, however, small as shown by chromatography.

The rate of degradation of the *amyloses* was expressed as (1) the number of bonds broken per initial molecule, and (2) $1/[\eta]$, both as a function of time.[2,6] For the products isolated after treatment with 0·2M-acid at 45°, the graphs of these functions were essentially similar to those for potato, and the extrapolated curves did not pass through the origin (cf. Fig. 3 of ref. 2). The linear portion corresponded, however, to a rate of only 0·1 bond broken per initial molecule per hour, which is 4—5 times slower than that for potato. For the M-acid at 16°, degradation was limited (*ca.* 0·05), and there was no evidence of any initial preferential breakdown.

Degradation of the *amylopectins* was calculated from the plot of $1/S$ *versus* t. For the reasons already given,[2] this must be a minimum rate. The samples isolated after treatment with 0·2M-acid at 45° showed an initial preferential breakdown, which was much larger than that for amylose and may well represent attack on amylopectin in the outer surface or amorphous regions (cf. ref. 2). The linear portion of the curve corresponded to a rate of about 0·4 (same units as above), which was slower than that for potato amylopectin. At 16°, the M-acid caused relatively little degradation (*i.e.*, a rate of *ca.* 0·07), although more than for the corresponding amylose, and in contrast to the results at 45° there was only limited initial preferential breakdown.

The effect of treating wheat-starch granules with 0·2M-acid at 45° could be explained by attack by acid in the manner already suggested for potato starch.[2] The calculated rates of degradation of the two components are, however, less than for potato. This may be related to a more compact structure for wheat starch, and thus an increased resistance to dispersion and fractionation.[1] The very limited accessibility of the granules is emphasized by the relatively negligible effect of M-hydrochloric acid at 16° on the molecular size of the components.

These studies suggest that there may be some fundamental difference in granular structure between tuber and cereal starches. Further methods of examining this are therefore being investigated.

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714. *Physicochemical Studies on Starches. Part XV.* The Action of β-Amylase on Glycogen as shown by Molecular-weight Distribution.*

By W. A. J. Bryce, J. M. G. Cowie, C. T. Greenwood, and I. G. Jones.

The changes in molecular-weight distribution occurring during the action of β-amylase on glycogen have been investigated by analysing the sedimentation diagrams obtained on ultracentrifugation of the original glycogen and two dextrins. The resultant distribution for the β-limit dextrin suggests that all the glycogen molecules in the sample, independently of molecular size, are hydrolysed to the same relative extent. Examination of an intermediate dextrin showed that during β-amylolysis, a mechanism involving degradation of all the polysaccharide molecules to the same extent appeared to be the most probable. The significance of these results is discussed.

Methods are available for converting sedimentation-velocity diagrams obtained on the ultracentrifugation of a polymer solution into molecular-weight distributions.[1-6] Little work of this type has been carried out on the components of starch. These studies are extremely valuable, however, as the pattern of action of any degradative agent can be followed *on a molecular basis*. As a preliminary to such studies on the starch components, we have analysed the changes in molecular-size distribution occurring during the action of β-amylase on glycogen in order to investigate the action-pattern of the enzyme and determine whether it has any degree of specificity with regard to the molecular size of the substrate. (This enzyme attacks the outer chains of glycogen and hydrolyses 45 ± 5% of the polysaccharide into maltose.) No previous work on this problem has been reported, although a similar study of phosphorylase action on glycogen has been made recently by Larner, Ray, and Crandall.[7] Glycogen has the advantage for this work that it possesses the most ideal sedimentation behaviour of all the starch-type materials; the concentration dependence of its sedimentation coefficient is small,[8] and this simplifies the calculations involved.

Our recent work [9] has shown the observed boundary gradient curve of glycogen to be very wide and dependent on the method of isolation. For an analysis of the distribution of sedimentation coefficients $g(S)$, a relatively narrow molecular fraction is preferable. This is not easily obtained from material of high molecular weight,[9] and hence a subfraction of alkali-extracted glycogen was used for these studies.

If the diffusion coefficient is negligible and the sedimentation coefficient (S) is independent of the concentration (c), the refractive-index gradient curve can be converted directly into a distribution of sedimentation coefficients $g(S)$ by the expression (cf. Bridgeman [1] and Baldwin [3]):

$$g(S) = (\mathrm{d}c/\mathrm{d}x)\omega^2 t x^3/c_0 x_0^2$$

Here, ω = angular velocity (in rads./sec.); t = time (in sec.) from the start of the sedimentation; x = distance (in cm.) of a point in the boundary from the axis of rotation; x_0 = distance (in cm.) of the meniscus from the axis of rotation; and c_0 = total concentration of the solution. However, the above conditions are obeyed by few polymers, and $g(S)$ has normally to be corrected for three boundary effects, (1) the spreading with

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time due to diffusion, (2) the anomalous apparent concentration of any individual molecular species due to heterogeneity—the Johnston–Ogston effect,[10] and (3) the narrowing due to the concentration-dependence of S. One can correct for diffusion by extrapolating an " apparent distribution " $g^*(S)$ *versus* $1/xt$ to infinite time.[2] One can correct for the other two effects either by extrapolating curves of $g^*(S)$ to infinite dilution,[4, 5, 11] or by correcting the curve of $g^*(S)$ at a single concentration for the dependence of S on c. The latter method [3] was adopted here. In view of the complex series of manipulations involved, the method is given in outline below.

Experimental

Glycogen Sample.—Glycogen was isolated from rabbit livers by extraction with hot 30% aqueous sodium hydroxide, and was purified and characterised as described elsewhere.[9] Sedimentation measurements showed that the sample was polydisperse, containing, in addition to the main component, both small and very large material (cf. ref. 9). A subfraction was obtained as follows: a 1% solution of glycogen in 0·1M-sodium chloride was centrifuged at 20,000 r.p.m. for 15 min. (Spinco ultracentrifuge) to remove very large material as gel. Cold ethanol was then slowly added to the supernatant liquid at 0°, to give a faint yet stable precipitate (approx. 30% by volume of alcohol was required). The precipitated glycogen was then removed by centrifugation at 1500 r.p.m. for 20 min. at 0°; under these conditions, the component of low molecular weight remained in solution.

Enzyme Preparation and Digest Conditions (with W. Banks).—β-Amylase was isolated from soya-beans as described by Peat, Pirt, and Whelan.[12] It contained only an insignificant trace of maltase, and no Z-enzyme as shown by experiments on potato amylose fractions.[13] α-Amylase was also absent as shown by the molecular size of the β-limit dextrin of both amylose [14] and the glycogen (see below). The activity of the enzyme in Hobson, Whelan, and Peat's units [15] was *ca.* 20,000 units/ml.

Glycogen (1 mg./ml.) was incubated with β-amylase in the presence of 0·2M-acetate buffer of pH 4·6 at 35°. Although conversion was complete within about 2 hr., the digest was left for 24 hr. before the enzyme was deactivated by heating it on a boiling-water bath for a few moments, and the glycogen-product (Found: 41% conversion into maltose) precipitated from solution with ethanol. After centrifugation, the residual β-limit dextrin was washed with alcohol and dried with ether. A polymer-product at an intermediate stage of β-amylolysis was isolated similarly (Found: 13·2% conversion into maltose).

Concentrations of glycogen were determined by hydrolysis to glucose. The latter and the amount of maltose liberated on β-amylolysis were determined by alkaline ferricyanide.[16]

Physical Measurements.—Sedimentation measurements were made as described earlier.[9, 17] The time representing the effective start of the sedimentation was obtained from the point where the curve of $\log_{10} X$ against t cut the time-axis at the value of $\log_{10} X$ for the meniscus.[17] The glycogen samples were dissolved in 0·1M-sodium chloride. Sedimentation runs for distribution analysis were carried out at 20,000 r.p.m., and a series of five photographs were taken at 6 min. intervals after the boundary had completely left the meniscus (the latter requiring about 10 min.). The concentration of glycogen was approx. 8 g./l. for these measurements.

Optical System of the Ultracentrifuge and the Measurement of the Photographic Plates.—The Spinco ultracentrifuge is equipped with a Philpot–Svensson [18] optical system, which gives the refractive index gradient curve directly. However, measurements of the height and area of the peak can be complicated by Fresnel fringes.[19] For this work, we have found an inclined bar to be more satisfactory than a wire.

When an appropriate base line has been fitted, the height (dn/dx) of at least 20 equi-spaced lamellæ at distances x_1, x_2, etc., throughout the refractive-index gradient curve has to be measured. These heights, when corrected for the angle of the inclined bar and the magnification factor of the optical system, give dn/dx values which are related to the total concentration (c) by the expression:

$$c = [\int (x/x_0)^2 \,.\, dn/dx \,.\, dx]/\Delta n$$

where x, x_0 have the values given above and Δn = specific refractive-index increment of the solute. (Trapezoidal integration is sufficiently accurate for this type of work.)

Our initial measurements were made directly from the photographic plates by using a two-dimensional travelling microscope (reading to 0·001 cm.) after the "vertical" traverse had been carefully aligned parallel to the meniscus. Heights of the two edges of the Schlieren pattern were measured. It was found most convenient to record these values and subsequent calculations directly on to a Remington–Rand printing calculator. Later measurements were made easier by printing an enlargement (9 ×) and tracing this on graph paper, corrections then being made for the additional magnification factor.

In both instances, base lines were fitted from the average of the two areas (A) under the peak and a knowledge of the concentration (c) of the solution, since

$$c = (\mathrm{A} \tan \theta / m_1 m_2 H_1 H_2 \Delta n)(x/x_0)^2$$

where θ = angle of the inclined bar in the optical system; m_1, m_2 = magnification of the cylindrical and camera lenses; H_1 = distance between the nodal point of the condensing lens and the inclined bar; H_2 = thickness of fluid column; Δn, x, x_0 are as defined above. The calculated and the actual concentrations agreed within experimental error.

Method of Determining the Molecular-weight Distribution.—(a) *The apparent distribution of sedimentation coefficients* $g^*(S)$. This function can be derived from the relations [20] $c = c_0(x_0/x)^2$ and $S = (1/\omega^2 t) \ln (x/x_0)$. It follows that $\mathrm{d}c = \mathrm{d}c_0(x_0/x)^2$ and $\mathrm{d}S = (1/\omega^2 x t)\mathrm{d}x$, and the combination of these equations gives $\mathrm{d}c_0/\mathrm{d}S = (\mathrm{d}c/\mathrm{d}x)(x/x_0)^2 \,.\, \omega^2 x t$. The curve of $\mathrm{d}c_0/\mathrm{d}S$ *versus* S is not a conventional distribution since the area under it is not unity but c_0. Normalisation of the function therefore gives the apparent distribution of sedimentation coefficients $g^*(S)$ as:

$$g^*(S) = (\mathrm{d}c_0/\mathrm{d}S)c_0^{-1} = \mathrm{d}c/\mathrm{d}x \,.\, (x/x_0)^2 \,.\, \omega^2 x t c_0^{-1}$$

This function was calculated for each sedimentation diagram for about 20 incremental values of x (*i.e.*, x_i etc.) by taking the corresponding values of $(\mathrm{d}n/\mathrm{d}x)_{x_i}$ for $(\mathrm{d}c/\mathrm{d}x)_{x_i}$ and $\Delta x \,.\, \sum_{x=0}^{x=\infty}(\mathrm{d}n/\mathrm{d}x)$ for c_0; the proportionality factors disappear in the quotient $(\mathrm{d}c/\mathrm{d}x)/c_0$. Conversion of the values of x_i into the corresponding values of sedimentation constant S_i[by $S_i = (1/\omega^2 t) \ln (x_i/x_0)$ after correction for viscosity and temperature] then enabled the graph of $g^*(S)$ versus S to be plotted for the different times of sedimentation.

(b) *Elimination of the diffusion effect.* From the graphs of $g^*(S)$ *versus* S, values of $g^*(S)$ for discrete values of $10^{13}S_i$ (*i.e.*, 10, 20, 30, etc.) were taken and plotted as $g^*(S_i)$ *versus* $1/x_i t$. A graphical extrapolation was then made to $1/x_i t = 0$ to yield values of the apparent distribution corrected for diffusion effects [$g'(S)$]. In agreement with Larner, Ray, and Crandall's results,[7] the data were best represented by straight lines, and all extrapolations were made on this basis. Our results were similar to those shown in Fig. 1 of ref. 7. The graph of $g'(S)$ *versus* S was thus obtained.

(c) *Transformation of* $g'(S)$ *into* $\mathrm{d}c/\mathrm{d}x$. Before corrections can be applied for the Johnston–Ogston effect,[10] the function $g'(S)$ *versus* S has to be transformed into $\mathrm{d}c/\mathrm{d}x$ *versus* x. The distribution equation can be re-written in this instance as:

$$\mathrm{d}c/\mathrm{d}x = g'(S) \,.\, x_0^2 c_0/x^3 \omega^2 t$$

where t is now chosen as the average time, *i.e.*, the time in the middle of the run.[7] Values of $(\mathrm{d}c/\mathrm{d}x)_{x_i}$ were therefore calculated from corresponding values of $g'(S)$. When values of S_i were converted into x_i by the expression $x_i = x_0 \exp(S_i \omega^2 t)$, the graph of $\mathrm{d}c/\mathrm{d}x$ *versus* x was obtained.

(d) *Correction for heterogeneity.* In order to correct for heterogeneity, the distribution curve $\mathrm{d}c/\mathrm{d}x$ *versus* x is divided into a number of equi-spaced lamellæ, and these are regarded as different components. Every molecular species i present in a given plane x_j changes in concentration at that plane, if its sedimentation coefficient S_i varies with the total concentration c_i. Baldwin [3] has shown that the change in concentration of the species (Δc_i) is related to the change in its sedimentation coefficient (ΔS_i) in terms of a parameter $(r/\omega^2 x)$, where

$$r/\omega^2 x = \ln (x_j/x_0)/\omega^2 t = S_i + c_i(\Delta S_i/\Delta c_i)$$

whence

$$\Delta c_i = c_i \Delta S_i / \{[\ln (x_j/x_0)/\omega^2 t] - S_i\}$$

To carry out these calculations, about 20 values of $\mathrm{d}c/\mathrm{d}x$ at a fixed increment, Δx, were tabulated against x, and the parameter $\ln (x/x_0)/\omega^2 t$ was calculated. The change in con-

centration of each of the components in each successive plane was then calculated in a stepwise manner by Baldwin's method.[3]

For the first lamella (x_1), only component 1 is present and therefore its concentration $c_1 = \Delta x \,.\, (\mathrm{d}c/\mathrm{d}x)_{x_1}$.

For the second lamella (x_2), the total concentration is $\Delta x \sum_{x=0}^{x=x_2} (\mathrm{d}c/\mathrm{d}x)$, which is an increase of $\Delta x(\mathrm{d}c/\mathrm{d}x)_x$. If $S = S_0(1 - kc)$ (see p.), the sedimentation coefficient of component 1 in this lamella $(S_1)_{x_2}$ therefore decreases by an amount $(\Delta S_1)_{x_2}$ given by $-kS_{01}\Delta x(\mathrm{d}c/\mathrm{d}x)_{x_2}$ where $S_{01} = S_1/(1 - kc_1)$. From Baldwin's work,[3] the corresponding change in concentration $(\Delta c_1)_{x_2}$ is thus equal to:

$$(\Delta c_1)_{x_2} = c_1 \,.\, (\Delta S_1)_{x_2}/\{[\ln (x_2/x_0)\omega^2 t] - (S_1)_{x_2}\}$$

The true concentration of component 2 (c_{02}) is thus greater than $\Delta x(\mathrm{d}c/\mathrm{d}x)_{x_2}$ by $-(\Delta c_1)_{x_2}$.

This calculation is repeated for all the components (i) in each lamella until the corrected concentrations (c_{oi}) of each are known. Then since $c_{oi} = \Delta x(\mathrm{d}c/\mathrm{d}x)_{xi}$, it follows that $(\mathrm{d}c/\mathrm{d}x) = c_{oi}/\Delta x$. Hence values of the corrected distribution function $g(S)$ were calculated from $g(S) = (c_{oi}/\Delta x) \,.\, (x/x_0)^2 \,.\, (\omega^2 x t/c_o)$, and the graph of $g(S)$ *versus* S obtained.

(c) *Correction for the concentration dependence of S; the extrapolation of g(S) to infinite dilution.* The distribution of sedimentation coefficients at infinite dilution $g(S_0)$ is derived from $g(S) \times (\mathrm{d}S/\mathrm{d}S_0)$, since $g(S_0) = c_0^{-1} \,.\, (\mathrm{d}c_0/\mathrm{d}S_0) = c_0^{-1} \,.\, (\mathrm{d}c/\mathrm{d}S) \,.\, (\mathrm{d}S/\mathrm{d}S_0)$. Here $\mathrm{d}S/\mathrm{d}S_0$ was obtained from tabular differentiation of S_{oi} and S_i values, S_{oi} being calculated from $S_i = S_{oi}(1 - kc_i)$ where $c_i = \Delta x \sum_{x=0}^{x=x_i} (\mathrm{d}c/\mathrm{d}x)$, as above. Values of $g(S_0)$ when plotted against the corresponding values of S_0 gave the true sedimentation coefficient distribution curve.

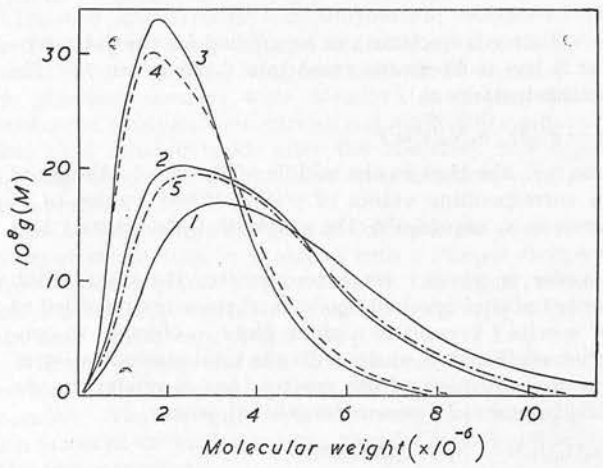
(f) *Calculation of the molecular-weight distribution curve.* Since the distribution of molecular weight $g(M)$ is given by:

$$g(M) = c_0^{-1}(\mathrm{d}c_0/\mathrm{d}M) = c_0^{-1}(\mathrm{d}c_0/\mathrm{d}S_0)(\mathrm{d}S_0/\mathrm{d}M)$$

values can be calculated from $g(S_0) \times \mathrm{d}S_0/\mathrm{d}M$. The results described elsewhere [9] enabled the value of $\mathrm{d}S_0/\mathrm{d}M$ to be obtained from differentiation of the relation (obtained by the method of least squares) between S_0 and M. This value was then utilised to calculate the curve for $g(M)$ against M.

Results and Discussion

The resultant molecular-weight distribution curves for the original glycogen (curve 1), the intermediate dextrin at 13% conversion into maltose (curve 2), and the β-limit dextrin (curve 3) are shown in the Figure. (It should be noted that these curves are weight- and



Molecular-weight distribution curves.

1, *Original glycogen.*
2, *Intermediate dextrin.*
3, *β-Limit dextrin.*
4, *Theoretical curve for β-limit dextrin (see text).*
5, *Theoretical curve for intermediate dextrin (see text).*

not number-distributions.) Although the original glycogen was subfractionated, it still possessed a very wide distribution—from about 1×10^6 to about 13×10^6—illustrating the difficulties inherent in obtaining sharp fractions of glycogen.[9]

Molecular-weight distribution curves obtained by the above methods are not absolute unless ideal polymers which are molecularly homogeneous are available: the form of the $g(S)$–S curve depends entirely on the relation between S and c, and this can be influenced by heterogeneity in molecularity. Also the transformation of the $g(S)$–S curve into the $g(M)$–M curve depends on $S = f(M)$, and this relation presents several difficulties experimentally when non-ideal samples are used: heterogeneous molecularity may influence the results, and there are often inaccuracies in measurements of the diffusion coefficients. [The method suggested by Williams and Saunders [4] for combining sedimentation-equilibrium and -velocity measurements appears to have many advantages in that diffusion measurements are avoided by using a double plot of integral distribution of M (from equilibrium measurements) and S (from velocity measurements).] For our calculations, we used the relation previously obtained [21] of $S_{20} = (S_{20})_0(1 - kc)$. Other results [9] have indicated that $dS/dM = (4{\cdot}39 \times 10^{-25})S^{-0{\cdot}59}$. The latter equation is necessarily not extremely accurate, but is as good as can be expected.[9] It would appear that an *absolute* molecular-weight distribution is not yet available by this method. However, the distributions obtained here are satisfactory for comparisons on a molecular basis of various types of degradation processes. (Since Larner and his co-workers [7] used different equations, no direct comparison is possible between the shapes of his distribution curves and those reported here.)

Mode of Action of the β-Amylase.—Although an accurate estimate of the enzyme concentration in these digests was not possible,[22] conditions were such that the substrate : enzyme ratio was high. The action pattern of the enzyme was investigated by comparing theoretical distributions calculated from the original for various mechanisms with those experimentally determined for the intermediate and limit dextrin. This was achieved by dividing the distribution curve into about 20 lamellæ of discrete molecular weight, and regarding each of these as a homogeneous polymer. If the attack of the β-amylase is random with regard to molecular size, then the decrease in molecular weight during β-amylolysis will be proportional to the number of non-reducing terminal units, *i.e.*, to the molecular weight, and the molecular-weight distribution for the limit dextrin (M_{LD}) will be simply related to that of the original (M_O) by $M_{LD} = (100 - c)M_O/100$, where c = the percentage conversion into maltose (*i.e.*, 41%).

Curve 4 is the result of such a calculation. Comparison with the experimental curve (3) shows agreement within experimental error. It appears that it is essentially correct that all glycogen molecules are degraded to the same relative extent after β-amylolysis; there is no appreciable preferential and more extensive degradation of material of either low or high molecular weight.

For the intermediate dextrin, a theoretical curve was first calculated on the assumption that 32% of the molecules over the whole molecular-weight range were converted to the limit of 41% of maltose (*i.e.*, the percentage necessary to account for the observed limit of 13%), while the remainder were unchanged. However, although the maximum in the resultant distribution was correct, the high-molecular-weight leading edge was very much higher than the experimental curve, and the amount of material in the molecular-weight range of 3—6 $\times$ 10^6 was too low, the differences in each case being outside experimental error. This mechanism was therefore not compatible with the experimental results. However, when the theoretical curve (curve 5) was calculated by assuming a 13% conversion of all molecular species, good agreement was obtained with the experimental curve. This suggests that during β-amylolysis, all glycogen molecules are degraded to the same extent independently of molecular size, and the enzyme does not in fact degrade one molecule completely before attacking another. Rather it appears that action must be random with regard to individual external chains. This is in agreement with unpublished kinetic experiments carried out by Mr. W. Banks. First-order kinetics are virtually non-existent, being obeyed for only the first 10% of the total reaction, and thereafter there is a gradual decrease in rate. This suggests that as β-amylolysis progresses, it

becomes increasingly more difficult to remove successive maltose units from any chain; such a mechanism implies that essentially all molecules will be degraded to the same extent throughout the reaction.

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782. *Physicochemical Studies on Starches. Part XVI.* The Molecular Weight and Apparent Molecular-weight Distribution of Rabbit-liver Glycogen.*

By W. A. J. Bryce, C. T. Greenwood, and I. G. Jones.

The effects of extraction with hot alkali and cold trichloroacetic acid on the molecular weight of rabbit-liver glycogen have been examined. Molecular weights have been obtained by both sedimentation–diffusion and light-scattering measurements. Apparent distribution curves of sedimentation coefficients for various glycogen samples have been calculated. Glycogen extracted by cold trichloroacetic acid appears to be more representative of native glycogen than that isolated by hot alkali. Difficulties in the sub-fractionation of glycogen are discussed.

Our recent physicochemical studies [1] of various glycogen samples have indicated that polydispersity † is quite common, and that good agreement between the molecular weights derived from sedimentation–diffusion and light-scattering measurements is unusual. Some of the factors involved for rabbit-liver glycogen are described here, as a preliminary to use of the results as standards for the hydrodynamic behaviour of branched glucosans. The molecular weight of this glycogen from sedimentation measurements has been reported [1,2] to be about 6×10^6, but in recent light-scattering work by Stetten, Katzen, and Stetten [3] molecular weights of $11—80 \times 10^6$ were obtained when extraction was with cold trichloroacetic acid, whilst alkaline extraction gave products of molecular weights $2—6 \times 10^6$. However, determination of the molecular weight of glycogen by only one physicochemical method is inadequate.[1] In the present work, we isolated the glycogen by different methods and investigated the products by (1) sedimentation-velocity measurements, to give an apparent molecular-weight distribution, and (2) turbidimetric measurements, to give the weight-average molecular weight ($\overline{M}_w$).

For use of glycogen as a standard for hydrodynamic behaviour, fractions with a narrow molecular-weight distribution are preferable. The sub-fractionation of glycogen has therefore also been examined.

Experimental

Isolation.—Livers from freshly killed rabbits were minced and divided into two portions. The glycogen in one portion was isolated by the classical Pflüger method of extraction with 30% sodium hydroxide solution and subsequent reprecipitation with ethanol and 80% acetic acid.[1] Glycogen isolated by this method is termed *OH*-glycogen. {Typical analytical figures were: glucose, 99% (on hydrolysis and estimation of the reducing power with alkaline potassium ferricyanide [4]); $[\alpha]_D^{16}$ +194° (*c* 0·2% in H_2O); conversion into maltose on β-amylolysis, 41%.} The other portion was extracted with trichloroacetic acid at 2° and the glycogen-product purified as described by Stetten, Katzen, and Stetten.[3] Glycogen isolated by this method is termed *TCA*-glycogen. {Typical analytical figures were: glucose, 98%; $[\alpha]_D^{16}$ +190 (*c* 0·2% in H_2O); conversion into maltose on β-amylolysis, 45%.}

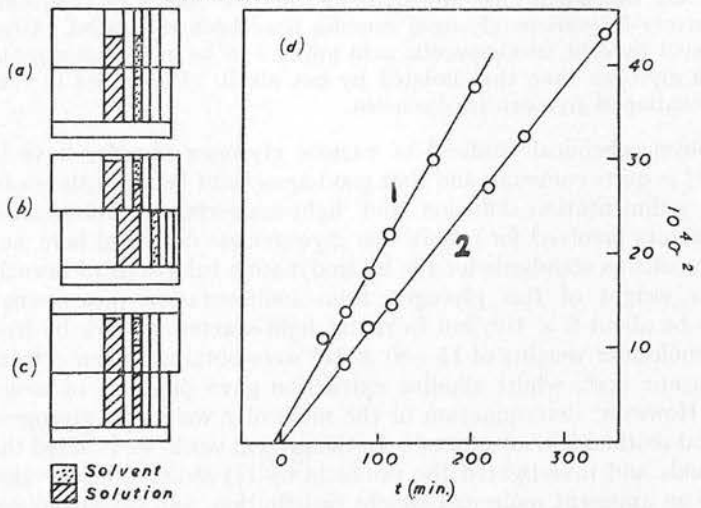
Sedimentation–Diffusion and Light-scattering.—These were carried out as described in Part XII of this series,[1] the solvent for the glycogen samples being 0·1M-sodium chloride.

* Part XV, *J.*, 1958,

† The term "polydisperse" is used to describe a polymer system containing more than one component, whilst "polymolecular" denotes a chemically homogeneous polymer having a variation in molecular weight.

Diffusion. These measurements were carried out in the Antweiler microelectrophoresis and diffusion apparatus at 20°. The concentration at a point x in a diffusion column is a function [5] of $x/\sqrt{t}$, and hence, if x is decreased by 10, the time required to reach a given concentration is reduced by 100. The duration of the experiment can therefore be considerably decreased if dn/dx can be measured at very small values of x. In practice, as there is usually an upper limit of dn/dx which can be measured, this can be achieved only by employing a cell with a short optical path, dn/dx for given values of x and t being then proportionately decreased. Hence, with a microcell a much shorter time is required for diffusion experiments. When solvent–solution boundaries are formed in the Antweiler all-glass diffusion cell by simply sliding one compartment over the other, the position of the initial boundary is obscured and readings of refractive index gradient at this point have to be interpolated throughout the measurements. This difficulty can be avoided by filling the cell as in Fig. 1*a*. The upper compartment is then moved to the position shown in Fig. 1*b*. Careful addition of more solution to the comparison compartment (by means of a micrometer syringe) will raise the

FIG. 1. (*a*), (*b*), *and* (*c*), *Antweiler diffusion cell* (*see text*). (*d*) *Typical graphs of* σ^2 *against t for* (1) OH-*glycogen* 1, (2) TCA-*glycogen* 1.



boundary from its interfacial position. The cell is then moved into position 1*c*, after removal of the residual solvent and its replacement by solution. In this manner, extremely sharp boundaries were formed. The refractive index gradient (dn/dx) in the liquid column was obtained either by arithmetical differentiation of the results from manually scanning the column with the Jamin interferometer, or by photography of the gradient obtained directly by the Schlieren optical-system attachment. For high concentrations of glycogen, the boundaries were too sharp to be measured satisfactorily by the interference method; in other respects the results from both methods of observation were the same.

Diffusion coefficients were evaluated by either the area–maximum ordinate method (D_a) or the second-moment method (D_m), where [6]

$$D_a = \left[\int_{-\infty}^{+\infty} y \,.\, dx\right]^2 \Big/ 4\pi t(y_{max.})^2 = A^2/4\pi t(y_{max.})^2$$

and

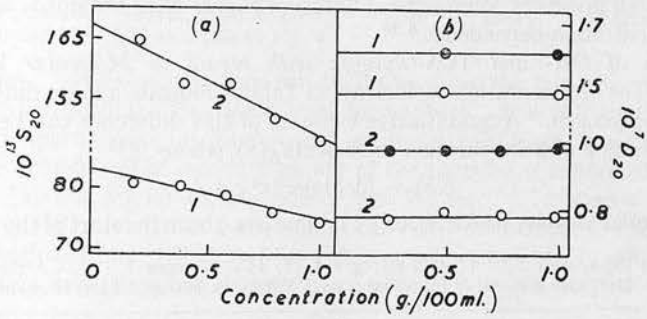
$$D_m = \left(\int_{-\infty}^{+\infty} x^2 y \,.\, dx\right) \Big/ \left(2t\int_{-\infty}^{+\infty} y \,.\, dx\right) = \sigma^2/2t$$

Here, x = the distance perpendicular to the boundary; $y = dn/dx$, the gradient of the refractive index; t = the time in seconds; σ^2 = the second moment of the curve; and A = the area. In all cases, the graph of σ^2 against t was linear and intercepted the t-axis at, or close (at a negative value) to, the origin (see Fig. 1). The time values used in calculations of diffusion coefficient were corrected for any apparent displacement of the zero time. Values are thought to be accurate to $\pm 3\%$ at any given concentration.

Ultracentrifugation. These measurements were made with a Spinco ultracentrifuge. Runs were usually carried out at 20,000 r.p.m. and at concentrations of 2·5—8·0 g./l. A speed of 8000 r.p.m. was necessary in studying the apparent molecular-weight distribution of *TCA*-glycogen. Sedimentation coefficients (S_{20}) were evaluated from measurements of the movement of the mode of the sedimentation diagram [*i.e.*, $(dn/dx)_{max.}$] in the usual manner. S_{20} values therefore represent the sedimentation coefficient of the molecular species apparently present in the largest amount.

Light-scattering. High-speed centrifugation could not be used to clarify the solutions before turbidity measurements, as rapid sedimentation of very large particles occurred (see below). Clarification was again achieved by filtration of concentrated solution through sintered glass (G4) under gravity. (Millipore filters and G5 filters were not satisfactory and tended to remove polysaccharide from solution.) For each sample, measurements were made at 4 or 5 concentrations in the range 1—10 $\times$ 10^{-5} g./ml. obtained by successive addition of the concentrated filtered solution to optically clear solvent. (The concentration of the original filtered solution was obtained by hydrolysis and estimation of the liberated glucose with

FIG. 2. (*a*) *Plot of* S_{20} *against c for* (1) OH-*glycogen* 1 *and* (2) TCA-*glycogen* 1.
(*b*) *Plot of* D_{20} *against c for* (1) OH-*glycogen* 1 *and* (2) TCA-*glycogen* 1; ● D_m *values*; ○ D_a *values*.



alkaline ferricyanide.[4]) This procedure gave reproducible turbidities and dissymmetries. Molecular weights were calculated from the equation: $Hc/\tau = 1/M(P_{90°}) + 2Bc/\boldsymbol{RT}$, where $H = 32\pi^3n^2(dn/dc)^2/3\lambda^4\boldsymbol{N}$; $(P_{90°})$ = a particle scattering factor, which was calculated on the assumption that the molecules were spherical; [1] B = the solute–solvent interaction parameter; dn/dc = the refractive index increment, which was taken [1] as 0·146 (c in g./ml.) for glycogen in 0·1M-sodium chloride at 546 mμ. Within experimental error, Hc/τ and I_{45}/I_{135} were found to be independent of c for the range of concentrations examined. The term $2B(\overline{P_{\theta}})_{\theta=0}c/\boldsymbol{RT}$ was therefore negligible.

The partial specific volume ($\bar{V}$) of glycogen was taken [1] as 0·62.

Subfractionation.—This was attempted by cooling a 0·1% solution in 15% (v/v) aqueous ethanol, and by differential centrifugation as described by Stetten, Katzen, and Stetten.[3] Stepwise addition of ethanol to aqueous solutions at room temperature was also tried.

RESULTS AND DISCUSSION

Concentration Dependence of S_{20} *and* D_{20}.—Sedimentation coefficients for rabbit-liver glycogen have been determined previously, but early investigations [7,8] were limited to only one concentration, Bridgman [7] stating that the concentration-dependence of S_{20} was less than the experimental error. However, recently we have confirmed Larner, Ray, and Crandall's results [9] that the concentration-dependence is real.[1] For the range of concentrations studied, $S_{20} = (S_{20})_0(1 - k_sc)$. Representative data are shown in Fig. 2*a*. By the method of least squares, values of k_s of 0·12 and 0·11 for *OH*- and *TCA*-glycogen respectively were obtained.

For diffusion coefficients also, early data [7,8] were restricted to one concentration. Bridgman's values [7] of $D_m = 1{\cdot}1 \times 10^{-7}$ for the majority of his samples were limiting values for time-dependent measurements. Ogston and his co-workers [8] gave values of 1·27—1·21 $\times$ 10^{-7}, whilst Larner and his co-workers' results [9] for samples of comparable

TABLE 1. *Molecular-weight data for* OH- *and* TCA-*glycogen.*

Method	Sedimentation–diffusion		Light-scattering			
Sample	$10^{13}(S_{20})_0$	$10^{-6}\,\overline{M}_{SD}$ [a]	$10^{-6}(\tau/Hc)$ [b]	I_{45}/I_{135} [b]	$10^{-6}\,\overline{M}_\tau$	$\overline{M}_\tau/\overline{M}_{SD}$
OH-Glycogen 1	84	3·1	13·6	1·70	19·0	6·1
			4·0 *	1·18 *	4·5 *	1·5
,, 2	86	3·3	—	—	—	—
,, 3 ‡	94	3·9	6·9	1·20	7·8	2·0
,, 4	95	3·9	7·1	1·20	8·0	2·1
TCA-Glycogen 1	168	9·4	38·5	2·00	62 †	6·6
			40·5	1·94	63 †	
,, 2	173	9·8	90	2·40	160 †	16·0
			91	2·40	162 †	
,, 3	163	9·1	—	—	—	—

[a] Calc. from data in Fig. 4. [b] Values at infinite dilution; I_{45}/I_{135} = dissymmetry ratio.
* Values after centrifugation at 20,000 r.p.m. for 15 min. (Spinco ultracentrifuge. Preparative rotor A.) † Independent determinations. ‡ Values from ref. 1.

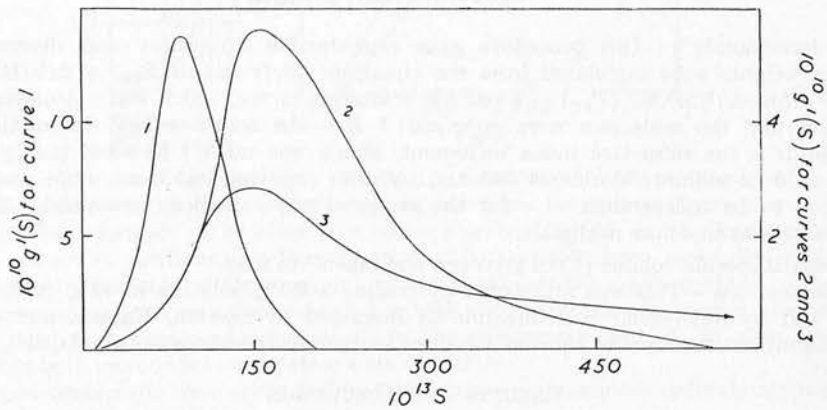
S_{20} were larger (1·3—1·5 $\times$ 10^{-7}) and possessed a definite, but variable, concentration-dependence. Our experimental results (see Fig. 2*b*) suggest that the dependence is negligible. In all instances, symmetrical diffusion curves were obtained, again indicating negligible concentration-dependence.[6,10]

Comparison of OH- *and* TCA-*Glycogen with regard to Molecular Weight and its Distribution.*—The sedimentation coefficients in Table 1 indicate a large difference between *OH*- and *TCA*-glycogen. A quantitative estimate of this difference can be obtained only from the distribution of sedimentation coefficients $g(S)$, where: [7,11]

$$g(S) = (\mathrm{d}c/\mathrm{d}x)\omega^2 t x^3/c_0 x_0^2$$

where ω = angular velocity (radians/sec.), t = time (sec.) from the start of the sedimentation,

FIG. 3. *Plot of* $g'(S)$ *against* S *for* (1) OH-*glycogen* 4, (2) TCA-*glycogen* 1, (3) TCA-*glycogen* 2[$g'(S) = 0$ *at* $S = 1400$; *the amount of this sample with* S *between* 600 *and* 1400 *is about* 25%].



x = distance (cm.) of a point in the boundary from the axis of rotation, x_0 = distance (cm.) of the meniscus from the axis of rotation, and c_0 = total concentration of the solution. An absolute distribution results only if diffusion is negligible and S is independent of c. Corrections for these effects can be made.[11,12] Here, Baldwin's method [11] has been employed to correct for diffusion and obtain apparent distributions $g'(S)$ (the calculations necessary for this distribution are detailed in Part XV). If $g'(S)$ is obtained at identical concentrations and the sedimentation behaviour of the samples is the same, the resultant curves should be comparable, although corrections for the Johnston–Ogston effect [11,13] and the concentration dependence [11] of S should ideally be applied. The $g'(S)$ curves shown in Fig. 3 emphasise the radical difference between *OH*- and *TCA*-glycogen (*e.g.*, only 8% of *OH*-glycogen 4 has $S > 150$, whilst *TCA*-glycogen 1 has 61% and *TCA*-glycogen 2 has 72%). Table 2 shows calculated values of the standard deviation,

mean (or weight-average) sedimentation coefficient, and skewness. The standard deviation for *OH*-glycogen 4 calculated from Baldwin's most recent work,[14] taking into account the concentration dependence of S, is the same (*i.e.*, 36*S*) as that calculated from the $g'(S)$ curve at 8·0 g./l. Both *TCA*-glycogens have a large positive skew; that for *OH*-glycogen is relatively small. The ratio[15] D_m/D_a (Table 2) from diffusion measurements indicated the increased polymolecularity of *TCA*-glycogen.

When molecular weights were calculated from S_{20} and D_m (to give $\overline{M}_{SD}$) for four samples, then $S_{20} \approx \overline{M}_{SD}^{0.63}$ (see Fig. 4). From Kuhn and Kuhn's results,[16] (1) $S \approx M^{\frac{1}{2}}$ for a matted coil, and (2) $S \approx M^{\frac{2}{3}}$ for a sphere, and hence glycogen may behave as essentially

TABLE 2. *Sedimentation coefficients and derived quantities from $g'(S)$ curves.*

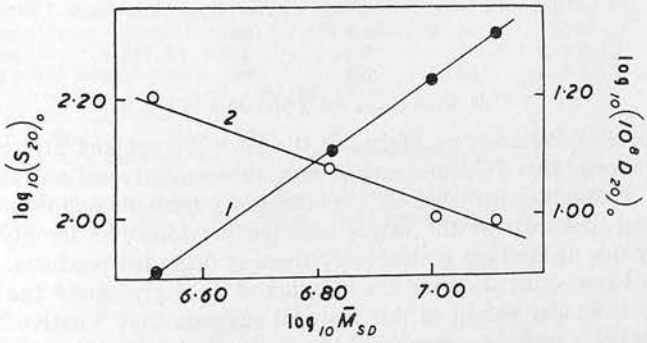
Sample	$10^{13}S_{20}$ [a]	$10^{13}S_m$ [b]	σ [c]	Sk [d]	D_m/D_a	$10^{-6}\overline{M}_{SD}$ [e]	$10^{-6}M$ [f]
OH-Glycogen 4	87	95	34	+0·23	1·08	3·9	5·0
TCA-Glycogen 1 ...	150	220	99	+0·70	1·27	9·4	18
,, 2 ...	150	417	310	+0·87	—	9·8	45

[a] Sedimentation coefficient as measured from the mode. [b] Calc. mean sedimentation coefficient [c] Standard deviation. [d] Skewness = (mean − mode)/σ (see, *e.g.*, Yule and Kendall, "Introduction to the Theory of Statistics," Griffin, London, 1950. [e] Values from Table 1. [f] Molecular weight calc. from S_m by assuming $k_s = 0{\cdot}12$ and data in Fig. 4.

spherical particles. Similar conclusions can be drawn from the fact that $D_{20} \approx \overline{M}_{SD}^{0.37}$ (see Fig. 4), an exponent of 0·33 being expected for a sphere. It is realised these relations are, at best, only approximate, as fractionated samples should have been used: this was not possible (see below). {The compact nature of the particles is confirmed by viscosity measurements. $[\eta]$ was 6·2 for *OH*-glycogen 1 and 7·1 for *TCA*-glycogen 1. (Units of concentration = g./ml.; solvent = 0·1M-sodium chloride; temperature = 22·5°.)}

Molecular weights from sedimentation–diffusion measurements on extremely polymolecular polymers are not simple averages, but depend[17] on the shape of the molecule

FIG. 4. *Plots of* (1) $\log_{10}(S_{20})_0$ *against* $\log_{10}\overline{M}_{SD}$, *and* (2) $\log_{10}(D)_{20})_0$ *against* $\log_{10}\overline{M}_{SD}$ *for glycogen samples.*



and the methods of evaluating S and D, and hence no direct correlation is to be expected with the results of light-scattering measurements ($\overline{M}_\tau$ values) shown in Table 1.

$\overline{M}_\tau$ for *OH*-glycogen is much less than that for *TCA*-glycogen. Differences between $\overline{M}_\tau$ for *TCA*-glycogens 1 and 2 are explained by the differences in skewness of the $g'(S)$ curves. The ratio $\overline{M}_\tau/\overline{M}_{SD}$ appears to give a qualitative measure of skewness of the distribution and values are given in Table 1. Molecular weights calculated by using mean sedimentation coefficients from the $g'(S)$ curve are more comparable with $\overline{M}_\tau$ values (see Table 2).

Stability of TCA-*Glycogen.*—The effect of various reagents on the sedimentation behaviour of *TCA*-glycogen was examined to investigate whether physical aggregation was occurring. Ultrasonic experiments (which will be described in detail elsewhere)

showed that under conditions which rapidly degrade amylopectin no appreciable change occurred in either S_{20} or the appearance of the leading edge of the sedimenting boundary. *TCA*-Glycogen also appeared to be stable to dilute acid and alkali at room temperature. After 72 hr., 0·5% solutions in 0·2M-potassium hydroxide and -acetic acid had the same S_{20} value as a control solution in 0·2M-sodium chloride, and there was no apparent change in the leading edge. When an aqueous solution (under air) was heated on a boiling-water bath, S_{20} was virtually the same after 1 hr. ($158S \longrightarrow 151S$), and even after 4 hours' heating, there was relatively little effect ($131S$). Changes in the leading edge were then apparent which did not appear to be reversible. Limited degradation or disaggregation must therefore have occurred.

The above experiments suggest that *TCA*-glycogen dissolves to form an essentially molecular dispersion. Further, $\overline{M}_\tau$ for the limit dextrin produced by the action of β-amylase had decreased by 50% compared with the 45% enzymic conversion into maltose. This is in agreement with Stetten, Katzen, and Stetten's results,[3] and suggests that aggregation was limited, as it appears unlikely that the extent of any aggregation, persisting after the 45% loss of weight on β-amylolysis, would be equivalent to that before treatment with enzyme.

However, when a 0·2% solution in 30% aqueous potassium hydroxide (under air) was heated on a boiling-water bath, S_{20} decreased rapidly and then remained constant ($168S \longrightarrow 100S$ in $\frac{1}{2}$ hr. $\longrightarrow 83S$ in 1 hr. $\longrightarrow 86S$ in 4 hr.). The sedimentation diagram was then indistinguishable from that of *OH*-glycogen. A similar effect was found when heating was in a nitrogen atmosphere. *TCA*-Glycogen appears to be alkali-labile, but oxidative degradation appears to be insignificant. *OH*-Glycogen must be a degradation product relatively stable to alkali.

TABLE 3. *Sub-fractionation of* TCA-*glycogen.*

Sample:	1			2			
Method:	Cooling to 6° and centrifugation			Differential centrifugation			
Yield (%)	$10^{-6}\,\overline{M}_{SD}$ *	$10^{-6}\,\overline{M}_\tau$	$\overline{M}_\tau/\overline{M}_{SD}$	Yield (%)	$10^{-6}\,\overline{M}_{SD}$ *	$10^{-6}\,\overline{M}_\tau$	$\overline{M}_\tau/\overline{M}_{SD}$
45	10·3	66	6·4	38	—	250	—
30	7·9	55	7·9	42	12·7	90	7·1
20	8·6	50	5·8	16	8·6	13·3	1·5

* Calc. from $(S_{20})_0$ value and data in Fig. 4.

Effect of Isolation Procedure on Molecular Weight.—We support Stetten, Katzen, and Stetten's conclusions [3] that *TCA*-glycogen is more representative of native glycogen than *OH*-glycogen. Extraction with hot 30% potassium hydroxide solution causes obvious degradation, and molecular-weight values reported previously [1,2] for glycogens isolated from tissues by this method are undoubtedly those of degraded products. Since degradation may also have occurred during the isolation of *TCA*-glycogen,[3] the extremely high weight-average molecular weight of this material suggests that " native " glycogen may well *not be amenable to study by conventional physicochemical methods* (compare, for example, ref. 18). The difficulties involved in the study of the size of " native " glycogen are obviously very great. It should be noted, however, that for bacteria [19] and yeasts [20] *TCA*-glycogens are smaller than *OH*-glycogens, probably because the acid has only limited access and only material of low molecular weight is extracted without prior alkaline cytolysis.

The polydispersity of *OH*-glycogens apparent on sedimentation measurements varied; some were monodisperse, whilst others had both a large and a small component. Any large component could be removed by centrifugation, no significant amount (<5%) of material being lost (see sample 1, Table 1), and reprecipitation often removed the smaller component. In view of results with trichloroacetic acid, we regard polydispersity in *OH*-glycogen as due to an artefact. It is of interest that Bridgman [7] found evidence of components of low molecular weight in some of his samples.

Our previous results [1] indicated that S_{20} for *OH*-glycogen was comparable with that for glycogen isolated by boiling water. In view of the degradative effect of alkali, glycogen in the tissues which is accessible to the solvent action of hot water must be comparable in size with the degraded product.[21]

Sub-fractionation of TCA-*Glycogen.*—The results of our experiments are shown in Table 3. No significant fractionation occurred with successive addition of alcohol. The methods suggested by Stetten, Katzen, and Stetten [3] gave limited sub-fractionation, but changes in $\bar{M}_\tau$ are due almost entirely to changes in very large material. In no instance was there any real narrowing of the apparent molecular-weight distribution as shown by the sedimentation diagrams. Sub-fractionation of *TCA*-glycogen is obviously very difficult in view of the large molecular sizes involved.

[*Added, September 8th,* 1958.—Stetten *et al.*[22] have recently reached essentially the same conclusions as ours concerning *TCA*-glycogen.]

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EFFECT OF OXYGEN-TREATMENT ON THE β-AMYLOLYSIS OF AMYLOSE*

By W. Banks, C. T. Greenwood and J. Thomson

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The method of isolation of amylose determines its extent of degradation by purified β-amylase. A limit of 65–85% conversion to maltose is found for amyloses prepared via complex-formation from a starch dispersion.[1–3] However, aqueous leaching of the granules gives a low molecular weight fraction of amylose which is completely hydrolysed enzymically, whilst the β-amylolysis limit of the residual high molecular weight material may suggest a randomly-situated barrier.[3]

β-Amylase has a high degree of specificity and any modification of the α-1-4-linked anhydroglucose units in the amylose chain will arrest the progress of the enzyme. Although it has been suggested that branching may be responsible,[4] the barrier could well be an artefact introduced during the isolation and fractionation of the starch.[5–7] Experiments to confirm this idea have involved passing oxygen through an alkaline solution of the amylose and measuring the β-limit of the products.[2,7,8] Such experiments have been complicated by the fact that alkali causes random degradation of the amylose in the presence of oxygen. This, in conjunction with the use of amylose having a β-limit of *ca.* 80% conversion, makes the unambiguous interpretation of the results impossible. If random degradation of the chain occurs (without the introduction of barriers), the observed β-limit will rise. However, if the random degradation is accompanied by the introduction of further barriers, any variation in β-amylolysis limit might occur. Further, the conditions in such experiments are not analogous to those occurring during fractionation. For these reasons we have studied the effect of oxygen on amylose fractions which were completely hydrolysed to maltose before treatment. Results of such studies are shown in Table I.

Table I

Effect of oxygen at 95° C. on linear amylose fractions

Amylose[a]	*Conditions* (i) pH[b]	(ii) *Time* (*mins.*)	*Initial* [η]	*Final* [η]	β-*limit*[c]
Potato 1	9·2	20	290	260	86
Potato 2	9·2	20	260	220	86
Potato 3	9·2	20	230	195	87
Iris germanica 1	9·2	20	190	120	86
,, ,, ,,	9·2*	20	190	180	98*
,, ,, ,,	7·0	20	190	130	91
,, ,, ,,	Water	120	190	160	95
Iris germanica 2	Water	120	150	125	93

[a] Potato 1=Redskin; Potato 2=Golden Wonder; Potato 3=Homeguard

[b] Buffered solutions

[c] Expressed as percentage conversion to maltose

* Control run with nitrogen atmosphere

* This is Part XVII in the Series "Physicochemical Studies on Starches"

It is obvious that treatment of amylose with oxygen in either neutral or weakly alkaline solution introduces barriers to β-amylolysis. Simultaneous slight degradation of the amylose chain also occurs.

The fact that barriers are introduced in neutral solution suggests that a similar process may be occurring during fractionation of starch. Confirmation of this idea is shown by the results in Table II, where the β-limit remains constant for the total amylose obtained on fractionation, although random degradation must have concurrently occurred. For an amylose—with a randomly-situated barrier-undergoing non-specific degradation, the observed β-amylolysis limit (L) at any stage in degradation will be related to the original β-amylolysis limit (L_0) by $L=100-\{(100-L_0)[\eta]/[\eta_0]\}$, where $[\eta_0]$ and $[\eta]$ are the limiting viscosity numbers of the original and degraded samples. (This relation is based on the fact that $[\eta]$ is directly proportional to molecular size.[9]) The difference between the observed β-limits and those calculated from the above formula (see Table II) suggests that the presence of oxygen during fractionation does in fact introduce barriers.

Table II

Introduction of barriers to β-amylase during fractionation of potato starch

Sample[a]	*Atmosphere during* (i) *fractionation*	and (ii) *recrystallisation*	[η]	β-*limit* (L_0)	*Calculated* β-*limit* (L)[b]
Ia	N_2	N_2	435	77*	77
Ib	O_2	N_2	380	75*	80
Ic	O_2	O_2	215	76*	89
IIa	N_2	N_2	470	85	85
IIb	air	N_2	350	86	89
IIc	air	air	300	85	90

[a] Sample I=Redskin; Sample II=Golden Wonder;

[b] See text

* Values obtained by courtesy of Drs. I. D. Fleming and D. J. Manners

In conclusion, we have confirmed that molecular oxygen is capable of introducing into amylose barriers to the action of pure β-amylase. (This is in agreement with the work of Gilbert[7] and Manners & Wright.[10].

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The Properties of Amylose as Related to the Fractionation and Subfractionation of Starch

by

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The Properties of Amylose as Related to the Fractionation and Subfractionation of Starch*

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(Eingegangen am 23. März 1959)

SUMMARY:

Factors which influence the apparent molecular size of amylose are discussed. The effect of various isolation procedures on the molecular size of the amylose from potato starch has been studied. Degradation occurs when sulphur dioxide saturated water is used to extract the granules; a method involving 0.01 M mercuric chloride is recommended. The properties of the amylose obtained on aqueous leaching of the granule are controlled by the pretreatment of the granules. This behaviour has been studied in detail. When a granular starch is treated with boiling 80% aqueous methanol, leaching results in the extraction of a subfraction of amylose which is hydrolysed completely to maltose under the action of β-amylase. Other procedures involving the action of boiling organic solvents on potato and wheat starches have been critically examined; degradation and modification of the properties of the amylose may then occur. The gelatinization of pretreated granules has been examined.

A series of subfractions of amylose has been obtained from barley and potato starches by leaching successively with water at increasing temperatures; the amylose remaining in the residual granule was then obtained by complete dispersion. These subfractions showed an increase in molecular size (by viscosity measurements) which was accompanied by a decrease in the percentage conversion to maltose on β-amylolysis. The implications of these results with regard to the structure of amylose are discussed. Various methods for swelling granules prior to dispersion have also been examined; pretreatment with liquid ammonia was found to be most satisfactory for aiding dispersion. The method of fractionation of potato starch by treating the granules with alkali has also been studied.

ZUSAMMENFASSUNG:

Einflüsse, die die Molekülgröße der isolierten Amylose ändern, werden besprochen. Die Wirkung verschiedener Isolierungsmethoden auf die Molekülgröße der Amylose aus Kartoffelstärke wurde untersucht. Es zeigt sich, daß beim Ausziehen der Körner mit wäßrigem Schwefeldioxyd Abbau stattfindet; eine Methode, die 0.01 M Quecksilber(II)-chlorid gebraucht, wird empfohlen. Die Eigenschaften der durch Extrahieren der Körner mit Wasser erhaltenen Amylose hängen von der Vorbehandlung der Körner ab. Dieses Verhalten wurde eingehend untersucht. Die Behandlung der Stärkekörner mit kochendem 80% wäßrigem Methanol zieht einen Amyloseanteil aus, der durch β-Amylase vollständig

*) This is Part XVIII in the Series "Physicochemical Studies on Starches"; Part XVII, Chem. and Ind. **1959**, in press.

zu Maltose hydrolysiert wird. Andere Verfahren, die kochende organische Lösungsmittel gebrauchen, werden kritisch untersucht; Abbau und Änderung der Amylose können dabei stattfinden. Die Verkleisterung vorbehandelter Körner wurde untersucht.

Eine Reihe von Amylosefraktionen wurde von Gersten- und Kartoffelstärken durch sukzessive Extraktion mit Wasser bei zunehmenden Temperaturen erhalten; der Amyloseteil, der in den Körnern zurückblieb, wurde am Ende durch vollständige Lösung isoliert. Diese Fraktionen zeigen ein Zunehmen der Molekülgröße (viskometrisch bestimmt) und ein begleitendes Abnehmen der prozentuellen Umwandlung durch β-Amylase. Die Bedeutung dieser Resultate in bezug auf die Struktur der Amylose wird besprochen. Die Wirksamkeit verschiedener Methoden zur Quellung der Körner vor der Lösung wurde auch untersucht; Vorbehandlung mit flüssigem Ammoniak war am geeignetsten. Die Fraktionierung der Kartoffelstärke durch Behandlung der Körner mit Alkali wurde studiert.

Introduction

The essentially linear amylose component of starch can be separated from the highly-branched amylopectin in several ways. The most satisfactory one involves complete dispersion of the granule into aqueous solution followed by precipitation of the amylose as a complex on the addition of a polar organic molecule. Aqueous leaching of the granule is apparently less efficient. These methods have been reviewed elsewhere[1]).

Although aqueous leaching does not completely separate amylose from amylopectin, the method is most important in fundamental studies of granular structure. Our recent work[2, 3]) has indicated that by combining a preliminary aqueous leaching with a complete dispersion of the swollen residual granules, it is possible to obtain sub-fractions of amylose, which differ fundamentally both in molecular weight and in the extent of enzymic degradation with β-amylase. Peat[4]), Hopkins[5]), Hassid[6]), and their collaborators have suggested that β-amylase degrades only about 70 % of amylose into maltose, but we have preferentially extracted an amylose fraction which undergoes complete enzymic degradation and a residue which had a lower conversion into maltose and may contain a *randomly-situated barrier* to β-amylolysis. Further, the initial fraction of amylose was smaller (degree of polymerization ($\overline{D.P.}$) 1000–2000 anhydroglucose units) than the residual amylose ($\overline{D.P.}$ ~3000–4000).

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In the work described here, the properties of various further subfractions of amylose obtained on leaching are discussed. It has been shown that the effect of leaching is governed to no small extent by the method of isolation and pretreatment of the granules. Methods which have been suggested for the pretreatment of granules as an aid to dispersion have therefore been critically examined. These methods include treatment with (1) 1 M-sodium hydroxide at 0 °C.[7]), (2) liquid ammonia[8]), and (3) boiling organic solvents[9]). Further, the effect of alkali as a fractionating agent[10]) has been studied.

Experimental

Isolation of starches

Starch was isolated from potatoes (vars. Homeguard, Epicure, and Redskin). Tubers were extracted with 0.01 M mercuric chloride in a Blendor, and starch obtained by filtration of the extract through muslin. The product was purified by repeated sedimentation in 0.1 M sodium chloride, and finally any traces of protein were removed by shaking a saline suspension with toluene[11]). The purified starch was then stored in saline under toluene at 2 °C.

Starch was isolated in a similar manner from barley (var. Ymer). The product was defatted by refluxing with 80% aqueous methanol, and stored under methanol.

Starch from wheat (var. Victor II) had been obtained previously by ARBUCKLE and GREENWOOD[3]). It had been isolated from defatted endosperm before being defatted by refluxing with 80% aqueous methanol and being stored under methanol.

Pretreatment of starches

If not used directly as prepared, *potato* starch was pretreated in one of the following ways:

a) the granules were refluxed with boiling 80% aqueous methanol; the method normally used to defat cereal starches.

b) as described by HODGE, MONTGOMERY, and HILBERT[8]), liquid ammonia (100 ml.) was added to starch (10–15 g.) in a DEWAR vessel. After standing for 15 mins., the mixture was poured into ethanol (500 ml.) and allowed to stand overnight for the ammonia to evaporate. The starch was then filtered and washed with ethanol before dispersion.

c) granules were treated with 1 M potassium hydroxide at 0 °C. with stirring for 10 mins., before careful neutralization and subsequent dispersion.

d) a 5% suspension of starch in 85% aqueous butanol or dioxane was placed on a water-bath and heated from 30 to 89 °C. over a period of 1 hr. The temperature was then

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maintained at this value for a further hour, before the mixture was cooled to room temperature, diluted with several volumes of ethanol, and the product obtained by filtration and washing with ethanol[9]).

Fractionation procedures

a) *aqueous leaching experiments.* Granules were leached at either 60 °C. for 10 mins., or 70 °C. for 60 mins., or 98 °C. for 8 mins.; a 0.5 % aqueous suspension being slowly stirred in a nitrogen-atmosphere. After cooling to room temperature, the residual swollen granules were removed by centrifugation and examined for their amylose-content. The supernatant was carefully filtered through a glass-sinter, before the addition of butan-1-ol. The butanol amylose complex formed on standing at room temperature was recrystallized with butan-1-ol.

In one series of experiments, granules were leached successively at 70 °C., 80 °C., and 90 °C. before the residual gelatinized granules were completely dispersed and the remaining amylose precipitated as the butan-1-ol complex.

b) *aqueous dispersion experiments.* Starch was dispersed in boiling water with vigorous stirring in a nitrogen-atmosphere for 1 hr. to form a 0.5 % solution. After cooling to 60 °C., thymol (1 g./l.) was added. The amylose-thymol complex, formed on standing for 48 hrs. at room temperature, was removed by high-speed centrifugation. The product was then re-dispersed and recrystallized as the butan-1-ol complex. Amylopectin was obtained from the supernatant liquor by freeze-drying after removal of thymol by extraction with ether.

c) *alkaline leaching experiments.* Starch was stirred at 0.6 % concentration in 0.5 M sodium hydroxide under nitrogen and at room temperature for 30 mins. After this time, the "gelatinized granules" were removed by centrifugation in polythene tubes at a mean force-field of 40,000 g. for 2 hrs. in the preparative rotor of the Spinco ultracentrifuge.

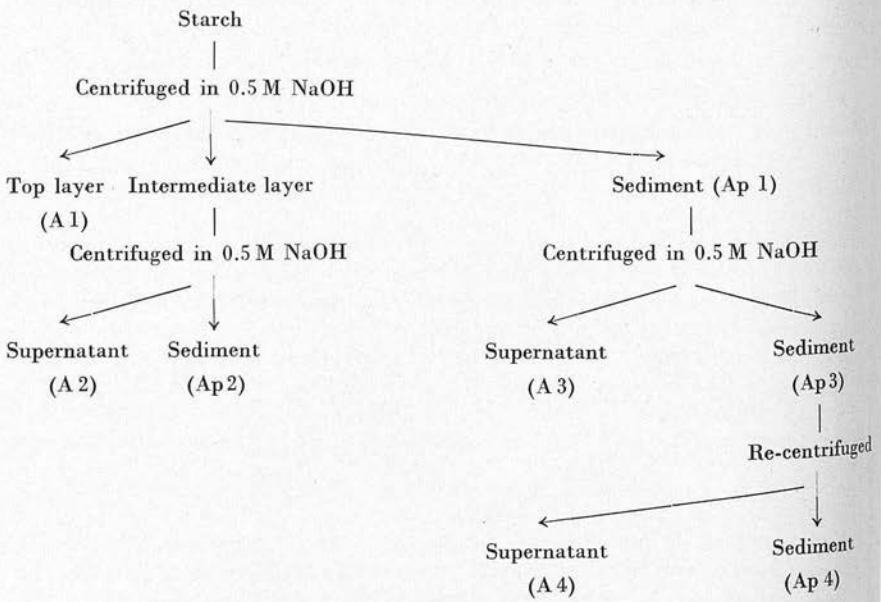

Fig. 1. Schematic outline of alkaline leaching of potato starch.

The top one-third of each tube was then removed by pipette, the solution carefully neutralized and the amylose precipitated by the addition of butan-1-ol. As the product was impure (see Discussion), two recrystallizations with butan-1-ol were made. The residual liquor and the sediment of gelatinized material was then re-extracted with alkali as indicated in Fig. 1, where amylose-containing supernatants are labelled (A), and amylopectin-containing sediments are labelled (Ap). The whole leaching experiment was repeated using a mean force-field of 20,000 g.

Characterization of starch components

To avoid retrogradation, amyloses were stored as their butan-1-ol complexes. Solutions were then prepared by dissolving the complex directly; concentrations being determined by hydrolysing aliquots and estimating the liberated glucose by oxidation with alkaline ferricyanide[12]).

a) *purity.* The purity of both starch components was determined from measurements of their iodine affinity using a semi-micro differential potentiometric apparatus described elsewhere[13]). For these measurements, the amylose-complex was dehydrated by repeated stirring with anhydrous butan-1-ol, dried *in vacuo* at 80°C. and then weighed directly. Titration conditions were: $[I'] = 0.01$ M; $p_H = 5.85$; temp. = 20°C.; $[\text{total free iodine}]_{max} = 1 \cdot 10^{-5}$ M. The iodine affinity is then 19.5% by weight for potato amylose[2]), and 19.0% for wheat amylose[3]).

b) *β-amylolysis limits.* The source of β-amylase was soya-bean flour, and the enzyme was purified by PEAT, PIRT, and WHELAN's method[4]). The preparation showed negligible maltase and no α-amylase activity and was free from Z-enzyme. (It has been found, however, that the preparation is contaminated with a large amount of low molecular weight protein and cannot be easily crystallized[14]). A sample of β-amylase contaminated with Z-enzyme was also prepared by eliminating the heat-denaturation step in the preparation.

Digests were prepared by dissolving amylose at 0.05–0.10% concentration in an acetate buffer at p_H 4.6, and amylolysis was carried out at 35°C. The amount of enzyme was such that the reaction was complete within a few hours, but digests were left for 24 hrs. before estimation of the liberated maltose. There was no change in the β-amylolysis limits on the addition of further enzyme. The concentration of polysaccharide in the digest was determined by hydrolysis of an aliquot. The glucose liberated on hydrolysis, and the maltose formed on β-amylolysis were independently determined by alkaline ferricyanide titration. This method has been found to be more satisfactory and reliable than that based on a copper-reagent of the SOMOGYI-type. The reagent is calibrated against glucose and maltose. Repeated calibrations against the standards are then unnecessary as the ceric sulphate solution can be simply standardized against ferrous ammonium sulphate when required; for a copper reagent a complete recalibration is always necessary when fresh reagent is prepared. A further disadvantage of the copper reagent is that when maltose is being estimated in the presence of amylose a blue coloration due to an amylose-iodine complex is present from the start of the titration, and this can cause difficulty. No such interference occurs with the ferricyanide method.

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[12]) J. M. G. COWIE and C. T. GREENWOOD, J. chem. Soc. [London] **1957**, 2658.
[13]) D. M. W. ANDERSON and C. T. GREENWOOD, J. chem. Soc. [London] **1955**, 3016.
[14]) W. BANKS and C. T. GREENWOOD, unpublished experiments.

c) *Limiting viscosity numbers* [η]. The viscosity of solutions of the components dissolved in 1 M potassium hydroxide were measured in a modified Ubbelohde viscometer at 22.5 °C. The kinetic energy correction was negligible. The average shear force was 1250 sec.$^{-1}$ [η] was obtained from the limiting value of η_{sp}/c at infinite dilution when c was expressed in g./ml. Our previous results[15]) have shown that at this shear force, the degree of polymerization ($\overline{D.P.}$) for potato amylose can be calculated from $\overline{D.P.} = 7.4 \cdot [\eta]$.

Results and Discussion

The apparent molecular weight of amylose

Reports in the literature for the molecular size of amylose are at variance, although there is evidence that the value depends on the botanical source of the parent starch. The apparent molecular size of a particular amylose depends primarily on whether fractionation is carried out by aqueous leaching or complete dispersion. Other factors can be, however, nearly as important. These include the effect of inadvertent degradation during (1) the isolation of the starch, (2) fractionation, and (3) the formation of derivatives. Further, in view of the wide variation of size in any sample of amylose, number-average ($\overline{M}_n$) and weight-average ($\overline{M}_w$) molecular weights differ considerably.

Laboratory-prepared starches yield, in general, less degraded components than commercial samples. This is particularly true for cereal starches isolated after steeping in sulphur-dioxide saturated water[9,16]). For this reason, we have always obtained this type of starch by a method avoiding acid-treatment[3]).

Degradation can occur during dispersion of a starch into aqueous solution (see below), but in addition, oxidative degradation of amylose always takes place when aqueous solutions are heated in the presence of air. An oxygen-free atmosphere during fractionations is therefore essential[2]).

The effect of inadvertent degradation of amylose during esterification has been considered by Greenwood and Robertson[11]) and Husemann and Bartl[17]). The latter authors have shown that both the degree of substitution and the reaction conditions influence the observed specific viscosity for a given sample of amylose.

Essentially undegraded amylose can be obtained only if all the above precautions have been observed. Unfortunately, these have been taken in relatively few instances.

Further, it is not easy to measure the molecular weight of amylose. In aqueous solution, the polysaccharide tends to retrograde, which compli-

[15]) J. M. G. Cowie and C. T. Greenwood, J. chem. Soc. [London] **1957**, 2862.
[16]) M. MacMasters and G. E. Hilbert, Ind. Engng. Chem. **36** (1944) 958.
[17]) E. Husemann and H. Bartl, Makromolekulare Chem. **18/19** (1955) 342.

cates light-scattering measurements in particular[18]). The study of derivatives presents the problems discussed above. For these reasons, we have preferred to characterize amylose samples by measuring their limiting viscosity numbers in 1 M potassium hydroxide. (Although degradation will occur on standing at room temperature in this solvent for long periods, the amount is negligible if the viscosity is measured immediately after solution of the sample; the latter occurs directly on the addition of alkali to the amylose-complex.)

Our previous results[15]) have indicated a linear relation between $[\eta]_{1250\ sec^{-1}}$ and $\overline{M}_n$ from osmotic pressure measurements on the corresponding acetate. It is realized that this relation is at best relatively crude, as the samples were unfractionated. However, it is thought that the calculated $\overline{D.P.}$'s are essentially correct from the results of unpublished sedimentation and diffusion measurements on the free polysaccharide in alkali. Although our results were obtained for potato amylose, we have used this relation for cereal amyloses also presuming that the error involved will not be large[3]).

Effect of isolation procedure on potato amylose

Experiments were made to investigate the effect of variations in isolation procedure – particularly the use of sulphur-dioxide steeping – on the amylose component of potato starch. Inactivation or inhibition

Table 1. The effect of variation in isolation procedure on the amylose component from potato starch, var. Homeguard

Isolation method	Fractionation method	% of amylose extracted[a])	I.A.[b])	$[\eta]$	$\overline{D.P.}_n$	β-amylolysis limit[c]
0.01 M $HgCl_2$	Dispersion	95	19.5	465	3500	84
	60 °C. leach	45	–	325	2400	80
C_2H_5OH	Dispersion	95	19.0	455	3400	78
	60 °C. leach	36	–	240	1800	79
SO_2-water	Dispersion	95	19.2	415	3100	84
(p_H 1.5)	60 °C. leach	42	–	310	2300	83
"Commercial"	Dispersion	95	19.5	335	2500	78
	60 °C. leach	42	–	261	2000	90

[a]) Calc. from iodine affinity of residual amylopectin.
[b]) I.A. ≡ iodine affinity.
[c]) Expressed as percentage conversion into maltose.

[18]) See e.g., E. F. Paschall and J. F. Foster, J. Polymer Sci. **9** (1953) 73, 75.

of enzymes during isolation can be achieved by either 0.01 M mercuric chloride, or ethanol, or saturated sulphurous acid. Results of experiments using these reagents for the extraction of starch are shown in Table 1, together with those for a commercial sample.

From the results of dispersion experiments, degradation appeared to have been caused by the sulphur dioxide, even though contact of the starch granules with the reagent was restricted to about 15 mins. before they were washed by repeated sedimentation in saline. This is in contrast to the steeping of about 24 hrs. often suggested for cereal starches; degradation in this time could obviously be extensive. The results for 0.01 M mercuric chloride and ethanol were similar, but the salt was used in subsequent extractions in view of the more rapid sedimentation of granules in this medium. The amylose from all preparations was again larger than that from the commercial sample. β-Amylolysis limits for these total amyloses were comparable. (It should be noted, however, that the value of 100% conversion into maltose reported earlier[2]) for the total amylose from the commercial sample is erroneous; the digests carried out for us then had been made with β-amylase containing Z-enzyme impurity, and the latter must have been incompletely inhibited in this instance.)

When all the starches were leached with water at 60 °C., material of smaller molecular size than the total amylose was extracted, but with the exception of the commercial sample, its β-amylolysis limit was little different from the total amylose. However, after one starch had been stored under methanol for 10 days (as in our previous work), aqueous leaching at 60 °C. yielded an amylose-fraction with $[\eta] = 285$ and a β-amylolysis limit of 96% conversion into maltose. The effect of pretreatment on the behaviour of the granule on leaching was therefore investigated in more detail.

Fractionation by aqueous leaching

1. Effect of pretreatment

Starch from several varieties of potatoes when extracted with 0.01 M mercuric chloride and stored under saline, did not yield a completely linear amylose-fraction on direct aqueous leaching. As previous results[2]) had been obtained on either samples of potato starch stored under methanol, or cereal starches which had been defatted with boiling 80% aqueous methanol[3]), the effect of treating *potato* starch with boiling 80% aqueous methanol was studied. Table 2 shows that after this treatment

a fraction of amylose, which was completely degraded on β-amylolysis, was obtained on aqueous leaching.

Table 2. Properties of amylose leached at 60 °C. from potato starch granules treated with boiling 80 % aqueous methanol

Variety	Treatment	% of amylose extracted[a])	% purity[b])	[η]	$\overline{D.P.}$	β-amylolysis limit[c])
Homeguard	None	40	100	250	1850	88
	MeOH	40	98	230	1700	99
Golden Wonder	None	40	101	280	2100	85
	MeOH	35	100	260	1950	98
Redskin	None	35	98	230	1700	88
	MeOH	35	99	250	1850	99

[a]) Calc. from iodine affinity of residual amylopectin.
[b]) Calc. from conversion into maltose under the concurrent action of β-amylase and Z-enzyme.
[c]) Expressed as percentage conversion into maltose.

Leaching at higher temperatures gave an increasing yield of amylose with a high $\overline{D.P.}$ and a *lower* β-amylolysis limit as reported before[2]). The latter was not due to contaminating amylopectin, as conversion into maltose was complete under the concurrent action of β-amylase and Z-enzyme. (Z-enzyme is capable of effectively removing the barriers to β-amylolysis which occur in some amylose-fractions, but is incapable of degrading amylopectin[4]).

The effect of boiling aqueous methanol on potato starch granules is unlikely to be that of simple defatting as there is a negligible amount of fat present. The most obvious effect is an alteration in the degree of crystallinity as the extent of swelling of the granule is decreased. This might render a portion of the amylose less accessible to the solvent-action of warm water. The problem of the variation in β-amylolysis limit concurrently observed is discussed below.

It has been found, in fact, that pretreatment of *any granular starch* with boiling 80 % aqueous methanol enables a subfraction of amylose which contains no barriers to β-amylolysis to be preferentially extracted on aqueous leaching.

MONTGOMERY and SENTI[9]) have also pretreated starches with boiling aqueous organic solvents. These authors suggested that this loosened the amylose-bonding in the granule so that $^3/_4$ was obtained on the first aqueous leaching at 98 °C. and the remainder subsequently. They also

suggested that *large* amylose was liberated first. Their procedure was therefore repeated using 85% aqueous butan-1-ol and 85% aqueous dioxane on potato and wheat starch. The results for potato starch (Table 3) cannot be directly compared with those of Montgomery and

Table 3. Properties of amylose fractions obtained from potato starch (var. Homeguard) after pretreatment

Pre-treatment	Fractionation procedure	% of amylose extracted[a])	% purity[a])	$[\eta]$	$\overline{D.P.}$	β-amylolysis limit[a])
None	98 °C. leach	Not successful	–	–	–	–
Butan-1-ol	98 °C. leach 1	55	99	240	1800	91
	98 °C. leach 2	0	–	–	–	–
Dioxane	98 °C. leach 1	45	100	100	750	55
	98 °C. leach 2	15	98	120	900	56
None	60 °C. leach	40	100	250	1850	88
Butan-1-ol	60 °C. leach	0	–	–	–	–
	70 °C. leach	25	100	160	1200	93
Dioxane	60 °C. leach	30	100	80	600	58
None	Dispersion	>95	99	450	3300	83
Butan-1-ol	Dispersion	>95	93	–	–	75
Dioxane	Dispersion	>95	94	–	–	59

[a]) As for Table 2.

Senti as these authors used a commercial sample. Such a starch behaves abnormally on leaching[2]). Our results show that treatment with boiling aqueous butan-1-ol and dioxane profoundly alters the swelling properties of the granule as the control did not retain its granular form under the leaching conditions. (The conditions[9]) are more drastic than those used in our earlier leachings at this temperature[15]).) The dioxane-treatment (commercial reagent) caused obvious degradation, and the product-amylose had an anomalous β-amylolysis limit. After the butan-1-ol-treatment, the first leaching extracted 55% of the amylose but contrary to Montgomery and Senti's results, no more was extracted on re-leaching. On leaching at 60 °C., a degraded product with a β-amylolysis limit of 58% was again obtained from the dioxane-treated sample, whilst no amylose was obtained from the butan-1-ol-treated sample. At a temperature of 70 °C. this latter starch then yielded a relatively small amylose-fraction with a high β-limit. The amylose from complete dispersions of the treated granules were found to be difficult to purify, and retained up to 10% of amylopectin.

Table 4. Properties of amylose fractions obtained from wheat starch after pretreatment

Pre-treatment	Fractionation procedure	% of amylose extracted [a]	% purity [a]	$[\eta]$	$\overline{D.P.}$	β-amylolysis limit [a]
None [b]	98 °C. leach	81	88	260	1900	69
Butan-1-ol	98 °C. leach 1	85	100	320	2400	64
	98 °C. leach 2	8	100	270	2000	65
Dioxane	98 °C. leach 1	81	100	200	1100	69
	98 °C. leach 2	8	99	180	1500	72
None	70 °C. leach	26	99	145	1100	98
Butan-1-ol	70 °C. leach	69	99	250	1850	70
Dioxane	70 °C. leach	57	100	150	1100	74
None	Dispersion	>95	96	260	1900	65
Butan-1-ol	Dispersion	>95	92	–	–	64
Dioxane	Dispersion	>95	90	–	–	68

[a]) As for Table 2.
[b]) This sample had been treated with 80% aqueous methanol during isolation.

Table 4 shows the results for comparable experiments on wheat starch. For these experiments, the dioxane had been carefully purified; it then behaved similarly to the butan-1-ol, but even so, degradative effects had not been completely eliminated. In agreement with MONTGOMERY and SENTI[9]), material from the first leaching at 98 °C. was slightly larger than that from the second leaching. However, even after several repeated leachings, some 7–10% of amylose remained in the residual granules. The control starch yielded impure amylose. Our values of $[\eta]$ for these amylose fractions are considerably larger than those reported by MONTGOMERY and SENTI[9]); this may be due to the latter authors not specifically purifying their reagents. On leaching at 70 °C., more amylose was extracted than for the corresponding control starch, with a consequent increase in $[\eta]$ and decrease in β-amylolysis limit. The amyloses from a complete dispersion were again not easily purified free from amylopectin.

The action of boiling aqueous butanol and dioxane on starch granules therefore renders the amylose more – but not completely – accessible to leaching. As a result, preferential extraction of essentially linear subfractions of amylose is made more difficult. The concurrent effect of apparent insolubilization of the amylopectin makes such treated granules unsatisfactory for subsequent conventional dispersion methods of fractionation. The effect of such pretreatment is different from that of

aqueous methanol. This may be related to the ease with which butan-1-ol forms complexes with amylose, and it is to be noted that MONTGOMERY and SENTI[9]) report the presence of a line on the X-ray diffraction patterns of such treated granules which was characteristic of an amylose-organic molecule complex.

However, all these methods of pretreating granules alter both the swelling power and the gelatinization temperature; there is no disruption of granules or loss in their birefringent properties. Microscopically it was observed for potato starch that swelling in warm water was most pronounced in untreated starch, and least in the butan-1-ol sample, with the methanol- and dioxane-treated samples behaving intermediately. When the corresponding gelatinization curves were obtained by using the hot-stage of a KOFLER microscope as described by SCHOCH and MAYWALD[19]), the range was as follows (where the intermediate value represents the temperature when 50 % of the granules were gelatinized): control: 58.5 °C. – 61 °C. – 63.5 °C.; methanol-treated sample: 57 °C. – 59.5 °C. – 62 °C.; dioxane-treated sample: 60 °C. – 61.5 °C. – 62.5 °C.; butan-1-ol-treated sample: 62.5 °C. – 64.5 °C. – 66 °C. The phenomena which are associated with swelling and gelatinization of starch granules are complex, but again it might be suggested that the degree of order in the granules has been altered.

2. Successive leaching of methanol-treated granules

Previously we have suggested[3]) from calculations based on the percentage of material which is completely hydrolysed by β-amylase, and the β-amylolysis limit of the total amylose, the existence of a subfraction of amylose with a randomly-situated barrier (i.e. a β-amylolysis limit of 50 %). This might be obtained by successive leaching followed by a complete dispersion of the residual granule. The results of such experiments on potato and barley starch are shown in Table 5.

It can be seen that it is not possible to obtain a high $\overline{D.P.}$ subfraction with an experimental 50 % β-amylolysis limit. The extent to which this is approached depends on the β-amylolysis limit of the total amylose, but the decrease in limit from the complete hydrolysis of the first fraction is accompanied by a large increase in molecular size as shown by changes in $[\eta]$. However, even if a random barrier exists in some of the amylose molecules, this apparent lack of separation could be due to the fact that

[19]) T. J. SCHOCH and E. C. MAYWALD, Analytic. Chem. **28** (1956) 382.

Table 5. Properties of amylose obtained by successive leaching followed by complete dispersion of granule

Starch	Procedure	% of amylose extracted[a])	% purity[a])	$[\eta]$	$\overline{D.P.}$	β-amylolysis limit[a])
Potato (var. Redskin)	58–60 °C. leach	35	99	250	1850	99
	63–65 °C. leach of residue	15	101	320	3200	82
	Dispersion of residue	50	98	570[b])	–	75
	63–65 °C. leach	45	100	290	2150	95
	Residue extracted at 20 °C.	15	97	350	2600	96
	Dispersion of residue	40	101	680[b])	–	71
Barley	70 °C. leach	22	101	93	690	96
	80 °C. leach of residue	17	100	138	1020	74
	90 °C. leach of residue 1	25	100	250	1840	69
	90 °C. leach of residue 2	18	100	298	2200	64
	90 °C. leach of residue 3	13	100	370	2750	63
	Dispersion of residue	5	99	380	2850	63

[a]) As in Table 2.

[b]) These values of $[\eta]$ are outside the range for which the relation $D.P. = 7.4 \cdot [\eta]$ was determined.

either (1) such a leaching procedure is inefficient, or (2) the distribution of molecules *not* having a barrier extends over the whole molecular weight range. Evidence on this point is obviously difficult to obtain, but unpublished determinations of the distribution of sedimentation coefficients have indicated that the range of molecular weights is large in all fractions.

The nature of the barrier to β-amylosis is in dispute[1]), but branching in the amylose may not be improbable as the enzymic resistance of fractions increases with increase in leaching temperature and consequent disruption of granular structure. However, the barrier could be an artefact[1]). It is to be noted that pretreatment with impure dioxane (see p. 206) did inadvertently introduce some barrier into all the amylose fractions. The problem is complicated by the inter-relation between β-amylosis limit and $\overline{D.P.}$, e.g. if the barrier is random and is due to branching then inadvertent degradation during isolation of the amylose would cause a rise in the observed β-amylolysis limit, whereas if the barrier is an artefact, any variation in β-limit could occur because such degradation might be accompanied by the introduction of more barriers. Further progress in this problem is unlikely to be made until the specificity of Z-enzyme has been determined, and such experiments are in progress.

Fractionation experiments involving dispersion

Some starches – of which those from cereals are the most common – are extremely difficult to disperse directly into aqueous solution. Autoclaving can be used but results in degradation[20]; some form of swelling pretreatment is better. Previously we have used the method[7] involving M-potassium hydroxide at 0 °C., but we have now extended our work to include the use of liquid ammonia[8]. Table 6 shows the properties of the amyloses from potato, wheat, and barley starch after such treatments.

Table 6. Properties of amylose from fractionation experiments involving alkaline- and liquid ammonia-pretreatments

Starch[a])	Pre-treatment	Purity[b])	$[\eta]$	$\overline{D.P.}$	β-amylolysis limit[b])
Potato 1	None	100	440	3200	77
	KOH	99	395	2900	–
Potato 2	None	100	380	2800	80
	$NH_3(l)$	100	410	3000	76
Wheat	None	96	260	1950	65
	KOH	99	258	1900	–
	$NH_3(l)$	101	280	2100	68
Barley	None	98	240	1800	73
	$NH_3(l)$	100	248	1850	73

[a]) Potato 1 = "Golden Wonder"; Potato 2 = "Epicure".
[b]) As in Table 2.

The results for potato starch show that liquid ammonia causes no degradation of the amylose and is preferable to alkali. Further, there has been no alteration in the β-amylolysis limit, and so the reagent does not introduce any anomalous barriers into amylose. These conclusions are confirmed for the wheat and barley starches.

Microscopic observation shows that after liquid ammonia treatment, the granules have lost their birefringent properties; all are swollen and some fragmented. The crystallinity of the granule has therefore been completely disrupted, but this has occurred without apparent degradation of the amylose. The effect on amylopectin is being currently studied.

After liquid ammonia treatment, wheat starch granules could be leached at 20 °C., when 65 % of the amylose ($[\eta] = 260$; β-amylolysis limit = 74 %) was accessible. For the corresponding ammonia-treated

[20]) R. S. Higginbotham and G. A. Morrison, Shirley Inst. Mem. **22** (1948) 148.

potato starch, leaching at 20 °C. gave an apparent dispersion. Addition of butan-1-ol did not, however, yield a precipitate. Warming to 60 °C. and the addition of thymol yielded a complex which contained 90 % of the amylose ([η] = 400; β-amylolysis limit = 81 %) in the granule. No explanation of this phenomenon can be advanced.

Pretreatment with liquid ammonia is easier and more satisfactory than the alkaline method, and hence we suggest that it should be used to pretreat any granular starch when complete dispersion is required.

Fractionation with alkali of potato starch

The properties of the various fraction obtained from potato starch by BAUM and GILBERT's method[10]) are shown in Table 7. The overall yield amounted to 83 % for Experiment I and 90 % for II; losses were primarily in the amylopectin as the total of amylose in the different fractions accounted for about 20 % of the original weight of starch. The yield of

Table 7. The properties of fractions obtained by fractionating potato starch, (var. Redskin) with alkali

Expt.	I (at 40,000 g. force-field)				II (at 20,000 g. force-field)			
Fraction	Weight (in mg.)	% of amylose[a])	[η]	β-limit[b])	Weight (in mg.)	% of amylose[a])	[η]	β-limit[b])
A1 (i)[c])	250	64	200	78	460	44	215	74
A1 (ii)[c])	–	100	470	86	–	100	435	85
A2	140	40	214	68	68	35	185	71
A3	100	7	185	58	92	6	220	60
A4	60	5	180	60	58	4	205	58
Ap1	Not isolated		–	–	Not isolated		–	–
Ap2	90	7	210	58	110	7	185	59
Ap3	Not isolated		–	–	Not isolated		–	–
Ap4	450	4	215	57	190	2	180	57

[a]) Calc. from (iodine affinity ÷ 19.5)·100.
[b]) Percentage conversion to maltose on β-amylolysis.
[c]) A1 (i) = sample before recrystallization; A1 (ii) = sample after recrystallization.

amylose in fraction A1 (1) was about 60 % for Expt. I and 80 % for II. The initial precipitates were very impure (64 % and 44 % of amylose, respectively), but on recrystallization twice as the butan-1-ol complex, amylose was obtained with a value of [η] (435–470) comparable to that of an amylose from a conventional dispersion of this starch (i.e. 480). Further, the alkaline treatment had not altered the β-amylolysis limit

of about 85 %. These amyloses represent, therefore, a portion of the *total* amylose present in the granule. Fractions A3 and A4, which precipitated on neutralization of the alkaline solution before the addition of butan-1-ol, were mainly amylopectin, and the two re-extractions of Ap1 therefore would appear to have resulted simply in solution of amylopectin. Recovery of amylopectin in the sediments was poor, being only about 50 % for Expt. I and 30 % for II (for the sum of Ap2 and Ap4 in each case). Further, the purity (2–7 % of amylose) was not comparable to that of amylopectin from a conventional dispersion when 0.1–0.5 % of amylose is usual[15]).

On the basis of these results, fractionation of potato starch by treating with 0.5 M sodium hydroxide is unsatisfactory: although, as claimed by BAUM and GILBERT[10]), separation can be achieved, it is not good. (It is to be noted that the "Blue Values" used by these authors are not accurate enough to characterize the purity of any amylopectin product[21]). We agree, however, that it is obvious that there are no bonds between amylose and amylopectin in the granule which resist cold alkali (compare ref. [22])).

These experiments imply that either (1) amylopectin is insoluble in alkali and amylose is leached from the granule, or (2) solution is achieved and the amylopectin undergoes simple preferential sedimentation. BAUM and GILBERT[10]) favour the idea of the insolubility of amylopectin. However, our aqueous leaching experiments suggest that complete removal of amylose is impossible without the complete dispersion of the granule, and yet fraction A1 (ii) has properties of the *total* amylose present in the granule. It would appear to us that complete solution of the granule has occurred and the components are sedimenting in the force-field used. From observations of the ultracentrifugal behaviour of solutions of the separated components in alkali[23]), it is known that appreciable sedimentation of amylopectin would occur in these force-fields; however, any "insoluble" material would be expected to be completely sedimented with these forces. The impure nature of fractions A (i) shows, in fact, that amylopectin is distributed throughout the medium. The differences in purity and yield between corresponding fractions in the two experiments are consistent with the forcefield used. The precipitation of amylopectin which occurred on the neutralization of supernatants through these experiments is thought to be due to reversible aggregation effects involving

[21]) R. S. HIGGINBOTHAM and G. A. MORRISON, Shirley Inst. Mem. **22** (1948) 141.
[22]) A. W. BAUER and E. PACSU, Textile Res. J. **23** (1953) 870.
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interaction between the phosphate groups – present in potato amylopectin – at the high salt concentrations employed. Unpublished experiments have indicated that this phenomenon (which is dependent on the p_H) is complex, and it is being further investigated.

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A COMPARISON OF THE STARCHES FROM BARLEY AND MALTED BARLEY

BY

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A COMPARISON OF THE STARCHES FROM BARLEY AND MALTED BARLEY

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Starch has been isolated from barley and malted barley of the same sample of cereal. The malted-barley starch had a higher apparent amylose content, a higher gelatinization temperature, and smaller granules than that from the original barley. Pre-treatment of the granules of both starches with liquid ammonia was necessary to achieve complete dispersion prior to fractionation into their component amylose and amylopectin. The amylose from the malted-barley starch was smaller in molecular size and had a higher β-amylolysis limit than that from the barley. The amylopectin fractions differed with regard to average chain length and β-amylolysis limit, but they were of comparable molecular size. Calculation showed the two amylopectins to have similar internal chain-lengths, but different external chain-lengths, that for the malted-barley amylopectin being smaller. This suggests that the malted-barley amylopectin had been degraded by β-amylase to a limited extent. Sub-fractions of amylose obtained on successive aqueous leaching of both granular starches showed a decrease in β-amylolysis limit with increase in molecular size. The properties of the components from malted-barley starch can be satisfactorily accounted for by assuming limited β-amylolysis of the amylopectin and very limited α-amylolysis of the amylose in the original barley starch.

INTRODUCTION

Although a comparison of barley starch and malted-barley starch should give an insight into the changes taking place in starch during malting, little work has been carried out on these lines. Barley starch usually contains about 22% of the linear amylose component associated with the

highly-branched amylopectin. Aspinall, Hirst & McArthur[3] have shown that malted-barley starch has a higher percentage of amylose and an amylopectin component with degraded external chains. The data of Hall *et al*,[12] are also consistent with an increase in the proportion of amylose.

Methods of fractionating starches.—Various methods can be used to fractionate starches into their components.[10,19] The most satisfactory one involves complete dispersion of the granules in boiling water (in an inert atmosphere to avoid degradation), followed by precipitation of amylose as an insoluble complex with substances such as thymol or butan-1-ol. This complex can be removed by centrifugation, and the amylose purified by reprecipitation. Amylopectin is left in the supernatant and can be obtained by precipitation or freeze-drying. Cereal starches are difficult, however, to disperse completely without some form of pre-treatment or prior swelling of the granule. This can be achieved with either cold 1-M potassium hydroxide, or liquid ammonia.

Whilst the method involving dispersion gives a quantitative yield of amylose, aqueous leaching of the granule at various temperatures provides useful information regarding the *fine-structure* of amylose; for not only does the amount of amylose extracted depend on the temperature, but so does its molecular size and β-amylolysis limit.

β-Amylolysis of amylose.—β-Amylase will degrade a linear α-1·4-glucosan by the stepwise removal of maltose units from the non-reducing end of the molecule. Now, although amylose was thought to degrade completely into maltose, recent work[16] has shown that with *purified* enzyme there is a limit of only 70–80% conversion. Another enzyme, Z-enzyme[16] (which is usually present in impure β-amylase preparations), is necessary before 100% conversion is reached. If a given sample of amylose is not degraded completely under the concurrent action of β-amylase and Z-enzyme, then amylopectin-impurity must be present. This is a reliable check of purity.

We have shown[8] that aqueous leaching of potato starch at low temperatures results in the preferential extraction of relatively low molecular weight molecules, which are completely converted to maltose with purified β-amylase. At higher temperatures, depending on the efficiency of the leaching, amylose of high molecular weight with a β-amylolysis limit approaching 50% can be obtained. On this basis, at least two types of amylose molecules are present; their proportions can vary from starch to starch.

In the work reported here, we have compared the general properties of the starch from barley (var. Ymer) and malted barley of the same sample of cereal. The two starches have also been fractionated by the above methods and the fine structures of both the amylose and amylopectin components studied.

Experimental

Estimation of starch in the kernels.—The procedure of MacWilliam, Hall & Harris[15] was adopted. The results showed that the percentage of starch was 64 for barley and 58 for malted barley (average of duplicate estimations).

Isolation and purification of starches.—Ground flour was extracted with water in a *Blendor* in the presence of 0·01-M mercuric chloride to inhibit enzymic action. The extract was filtered through fine muslin and the filtrate centrifuged to yield crude starch. The residue was re-extracted twice, after which the yield of starch was negligible. The crude starch was purified free from protein by shaking a saline suspension with toluene. This procedure[11] will denature and remove protein by purely physical means and so avoid any inadvertent degradation of starch. The purified starches were then refluxed with boiling 80% aqueous methanol to remove any residual traces of fats. Each then gave 99% glucose on hydrolysis. (Found % N = 0·017 for barley; % N = 0·024 for malted barley.)

Iodine affinity measurements.—The amount of amylose in a starch can be calculated from the amount of iodine that the starch binds. This can be measured by either colorimetric or potentiometric methods, but the latter is the more accurate. Here, a semi-micro differential titration technique has been used[1]; a starch-iodide-buffer solution and a control iodide-buffer solution are arranged as opposing half-cells connected by a salt-bridge, and the potential difference between the two is measured with a valve electrometer. After iodine has been added to the starch solution, the amount remaining unbound can be found by adding iodine to the control solution until there is no potential difference. The amount of bound iodine is then found

by difference, and curves of the type shown in Fig. 1 are obtained. The "iodine affinity" (I.A.) of a starch or starch component is found by extrapolating the limiting portion of the iodine titration curve to zero free iodine concentration (see Fig. 1). The

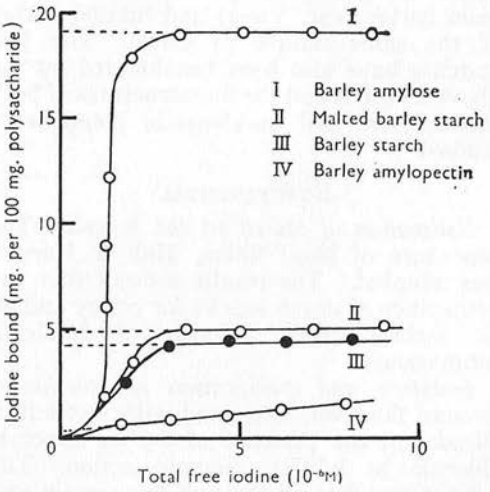

Fig. 1.—Typical potentiometric iodine titration curves for starch products. Extrapolations (– – – – –) yield "Iodine affinities."

percentage of amylose in any sample is then 100 (I.A. for sample)/(I.A. for pure amylose). Under the experimental conditions ([I′] = 0·01 M; pH = 5·85; temperature = 20° C.), I.A. for pure barley amylose was 19·0%.

Gelatinization temperature.—The gelatinization temperature is that at which granules lose their birefringent properties when heated in a swelling medium, usually water. For a given sample, this normally occurs over a range of some 5° C. A simple but accurate method of measuring the gelatinization temperature is to use the hot stage of a *Kofler* melting-point apparatus.[20] A drop of aqueous suspension of starch surrounded by oil was placed on a microscope slide and covered with a cover-slip. At various temperatures, the number of granules which had lost their birefringent properties was counted. Typical results are shown in Fig. 2.

Granular size distribution.—Photomicrographs (50 ×) of aqueous suspensions were enlarged to give a total magnification of about 480 diameters. At least 500 granules of each sample were then measured with a ruler to the nearest 1 mm. The granules of both starches varied in shape from spherical to

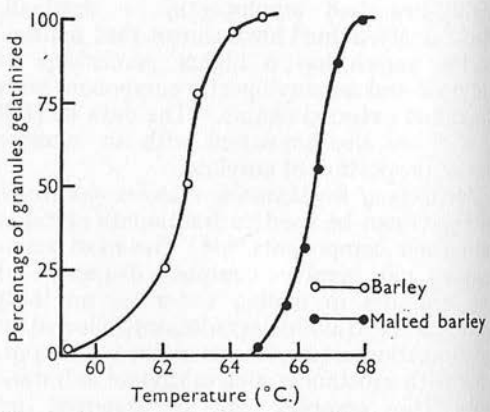

Fig. 2.—Percentage of granules gelatinized in water as a function of the temperature.

ovoid, and hence granules were characterized by their diameter for the spheres, and their major axis for ovoids. Number-percentage distribution curves were constructed from the resultant histograms as shown in Fig. 3*a*, and the corresponding weight-percentage distributions calculated assuming the density was independent of the granular size and that the ratio of major to minor axis did not vary. It is realized that the resultant curves (Fig. 3*b*) may not be extremely accurate, but they should be of the correct shape. Number-average and weight-average granule

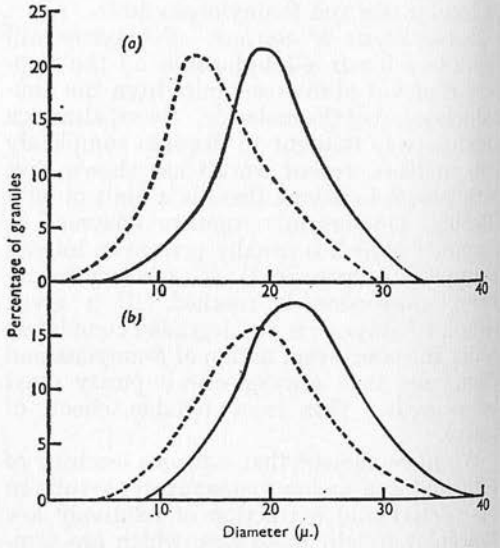

Fig. 3.—Distribution curves for (*a*) number-percentage, and (*b*) weight percentage of granules.
———— Barley starch
– – – – Malted barley starch.

diameters were then calculated from $\Sigma n_i d_i / \Sigma n_i$ and $\Sigma n_i d_i^4 / \Sigma n_i d_i^3$ where n_i = the number of particles of diameter d_i.

Pretreatment of starch.—Starches were pretreated with liquid ammonia by Hodge, Montgomery & Hilbert's method.[13] Liquid ammonia (100 ml.) was added to starch (10–15 g.) in a Dewar vessel. After 15 min., the mixture was poured into ethanol (500 ml.) and left overnight until the ammonia had evaporated. The starch was then filtered and washed with ethanol before dispersion.

Fractionation of starch by dispersion.[6]—Starch or pretreated starch (7·5 g.) was dispersed with vigorous stirring in water (2 litres) at 98° C. in a nitrogen atmosphere. This temperature was maintained for 2 hr., after which the solution was allowed to cool to 60° C., and thymol (3 g.) was added. After standing at room temperature for 48 hr., the amylose-thymol complex was removed on the *Sharples* supercentrifuge, and was purified by two reprecipitations as the butan-l-ol complex. The amylopectin was obtained by freeze-drying the supernatant liquor. (Thymol was removed by refluxing the solid with methanol.)

Fractionation of starch by aqueous leaching.[6]—A 0·2% aqueous suspension of starch was leached at 70° C. in a nitrogen atmosphere for 1 hr. After cooling to room temperature, the mixture was centrifuged. The supernatant was then carefully filtered through a SG4 filter to remove any insoluble material, before the addition of butan-l-ol. The amylose-butan-l-ol complex was recrystallized. The sediment was re-extracted successively at 80° and 90° C., before the residue was dispersed completely in boiling water and fractionated as above.

Measurements of limiting viscosity number $[\eta]$.[7]—$[\eta]$ was determined from the relation $[\eta] = \lim\ (\eta_{sp}/C)$ as $C \rightarrow O$. Viscosities were measured in a modified *Ubbelohde* viscometer at 22·5° C. using 1-M potassium hydroxide as a solvent. This type of viscometer allows dilutions to be made *in situ*. Concentrations were estimated by hydrolysis of aliquots and estimation of the liberated glucose by alkaline ferricyanide, and were expressed as g. per ml. An estimate of the degree of polymerization ($\overline{D.P.}$) for amylose fractions was obtained from the relation:[6]

$$\overline{D.P.} = 7{\cdot}4 \times [\eta],$$

found for potato amylose. Such values, although not extremely accurate, are likely to be of the correct order of magnitude.

β-Amylolysis experiments.—Purified β-amylase was prepared from soya bean flour by Peat, Pirt & Whelan's method,[16] which includes deactivation of Z-enzyme by a heat-treatment of the protein in acid media. The enzyme preparation showed no maltase, α-amylase, or Z-enzyme activity. A sample of enzyme containing β-amylase and Z-enzyme was also prepared by eliminating the heat treatment. Digests were carried out in acetate buffer (pH = 4·6) at 35° C. The concentration of enzyme was such that the reaction was virtually complete inside a few hours, but the digests were left for 24 hr. before concentrations of polysaccharide and liberated maltose were independently estimated by alkaline ferricyanide.[9]

Estimation of length of unit-chain of amylopectins.—Average lengths of unit-chain of the amylopectins were calculated from the yield of formic acid liberated on oxidation with sodium metaperiodate at 2° C. by Potter & Hassid's method.[17] The formic acid was determined potentiometrically; in agreement with Potter & Hassid, oxidation was found to be complete in 24 hr. A correction was made for the presence of any amylose.

Subfractionation of amylopectin.—Liquid-ammonia-pretreated starches were dispersed in boiling water, and the amylose removed as the thymol-complex in the usual manner. The amylopectin-containing supernatants obtained on centrifugation were shaken with ether to remove thymol, and solid sodium chloride was added to give a concentration of 0·3%. Ethanol was added to give a 20% (v/v) solution and the mixture was stored at 0° C. for 12 hr. The supernatant-liquor was then decanted from the precipitated gel-material. This procedure was repeated for ethanol concentrations of 30% and 50%, and each precipitate was dissolved in water and freeze-dried. The residual liquor was reduced in volume, dialysed to remove salt, and freeze-dried.

Discussion

The percentage of starch in the malted-barley kernels was less than that in the original barley; the loss represented about 16% of the initial weight of starch. This value is comparable to that of 18% reported by

Hall *et al.*[12] A quantitative yield of starch cannot be obtained for cereals without drastic chemical treatment of the grains. Here, the starches were isolated without the use of reagents likely to cause chemical degradation of the starch components. Although this necessitated incomplete removal of starch, the products should be comparable, and more importantly their components should be undegraded.

Properties of granular starches.—Table I summarizes some of the properties of the two starches. The malted-barley starch possessed a higher amylose content, a higher gelatinization temperature and smaller granules than that of the original barley. Fig. 1 shows that there was a significant increase in the iodine affinity of the malted-barley starch which corresponded to an increase in the amylose content of 22·4 to 26%. The value is in agreement with that from previous work.[3,12] The phenomenon of gelatinization is complex; the percentage of amylose, the size of the granules, the molecular size of the components and the corresponding degree of crystallinity in the granule may all influence swelling and the subsequent gelatinization.[20] However, the observed higher gelatinization temperature for the malted-barley starch (Fig. 2) may be related to both its smaller granular size and its higher amylose-content (*cf.* ref. 20). The distribution curves (Fig. 3) show that the granules from the malted-barley are significantly smaller than those from the barley. As both starches were in equilibrium with water when examined, these differences must be significant and are not due to the environment.

Fractionation experiments by aqueous dispersion.—When the components were isolated from an aqueous dispersion of the original untreated granules, it was found that the yield of amylopectin from both starches was only about one-third of the theoretical; the initial amylose complex was very impure. A similar effect was found by Aspinall *et al.*[3] These results confirmed our previous experience with cereal starches,[2] and showed that some form of pretreatment of the granules was essential to achieve complete dispersion. We used the method involving liquid ammonia[13] (solution could have been obtained by autoclaving, but this procedure causes degradation[14]). From the results of control experiments on potato starch and other cereal starches, we have shown that this procedure—although resulting in complete disruption of the granular structure as shown by loss of birefringent properties—causes *no* degradation of the components. The properties of the resultant fractions are shown in Table II. Although the amylopectin from the barley starch was reasonably pure, that from the malted barley (amylopectin 1) was only 75% pure. When the amylopectin solution was left in the presence of thymol for a week at room temperature, there was no further precipitate that could be removed on the *Sharples* supercentrifuge. However when butan-l-ol was added, the mixture warmed to 60° C., and allowed to cool to room temperature, more amylose-complex was formed. The resultant amylopectin (amylopectin 2) was then satisfactorily pure. It is not known why butan-l-ol should have caused further amylose-complex formation.

TABLE I

PROPERTIES OF GRANULAR STARCHES

Sample	Starch in kernel (% dry weight)	Iodine affinity (%)	Amylose (%)	Gelatinization temperature (° C.)	Size of granules* (μ.)
Barley	64	4·25	22·4	59·0–64·5	(i) 21, (ii) 24
Malted barley ..	58	4·95	26·0	64·8–67·5	(i) 17, (ii) 21

* Values for (i) number-average, (ii) weight-average diameters.

All the amylose fractions were pure as shown by their iodine affinities and their conversion limits to maltose under the concurrent action of β-amylase and Z-enzyme. The barley amylose was larger in molecular size and had a lower β -amylolysis limit than that from the malted barley – particularly fraction 2 (which amounted to 15% of the amylose present in the starch).

The amylopectins also differed with respect to both the average length of unit chain (calculated from the amount of formic acid

TABLE II

PROPERTIES OF COMPONENTS OBTAINED BY AQUEOUS DISPERSION OF THE STARCHES

Component	Iodine affinity (%)	Purity (%)	Limiting viscosity no. [η]	Degree of polymerization	β-Amylolysis limit*	Unit chain length
Barley amylose	19·0	>99	240	1,800	(i) 72, (ii) 98	—
Barley amylopectin	0·38	98	188	—	(i) 58, (ii) —	25·5
Malt amylose 1	19·0	>99	200	1,480	(i) 77, (ii) 98	—
Malt amylopectin 1	4·8	75	—	—	(i) —, (ii) —	
Malt amylose 2†	19·0	>99	115	850	(i) 90, (ii) 101	—
Malt amylopectin 2†	0·74	96	146	—	(i) 48, (ii) —	18·4

* Expressed as percentage conversion to maltose under the action of (i) β-amylase, and (ii) β-amylase + Z-enzyme.

† These samples were obtained from amylopectin 1 (see text).

liberated on oxidation with sodium periodate) and the extent of conversion to maltose on β-amylolysis (see Table II). The chain length of 25–26 anhydroglucose units found for barley amylopectin is comparable to that for other amylopectins,[10] but the malted-barley amylopectin had a chain length of 18 in agreement with Aspinall *et al.*[3] Also the β-limit of 48% conversion to maltose observed for the malted-barley amylopectin is lower than that of 58% for the barley sample. β-Amylase can only degrade the exterior chains of amylopectin and its action stops at 2 or 3 glucose units from an inter-chain linkage. The average *external* lengths can therefore be calculated to be 17–18 and 10·5–11·5 glucose units for the barley and malted-barley amylopectins respectively.

The corresponding average *internal* chain-lengths of 7·5–8·5 and 7–8 glucose units are therefore comparable. In an attempt to show whether the low chain-length observed for the malted-barley amylopectin was due to a mixture of enzymically degraded and non-degraded polysaccharides, sub-fractionation of amylopectin by preferential precipitation with ethanol was carried out. The results in Table III show that this procedure did not result in fractions with significantly different average chain-lengths or β-amylolysis limits.

The measurement of the molecular weight of amylopectin is difficult in view of the very large values involved. Viscosity measurements are insensitive; but sedimentation velocity measurements can give some idea of the relative molecular sizes.[5] When the two amylopectins dissolved in 0·2-M potassium hydroxide were examined in a *Spinco* analytical ultracentrifuge at concentrations between 0·1 and 0·5 g. per 100 ml., comparable sedimentation coefficients (S_{20}) were observed. The graph of S_{20}^{-1} against C extrapolated to about 250×10^{-13} c.g.s. This indicated that the amylopectins were both large and comparable in size.

TABLE III

PROPERTIES OF SUBFRACTIONATED AMYLOPECTINS

Amylopectin from:	Ethanol in mixture (%)	Amylopectin fraction (%)	Unit chain length	β-Amylolysis limit
Barley	20	70	25·8	58
	30	2	25·6	—
	50	3	25·4	—
	>50	25	25·5	59
Malted barley ..	20	74	18·1	48
	30	2	17·7	—
	50	3	17·6	—
	>50	21	17·8	48

Successive leaching experiments.—The results of these are shown in Table IV, and both starches show the same general pattern as that previously observed.[8] All the amylose subfractions were essentially pure, as shown by the high conversion to maltose on the concurrent action of β-amylase and Z-enzyme; therefore the variation in β-amylolysis limit reflects variations in the properties of the amyloses. For both starches leaching at low temperatures yields material which is completely degraded by β-amylase, whilst with increase in temperature, material with a lower β-limit is obtained. Previously, from calculations based on the percentage of the completely hydrolysed fraction and the β-limit of the total amylose present in the starch, we have postulated[2] the existence of material which has only a 50%-limit. The experiments reported here lend support to this idea. Obviously the separation of amylose by leaching must be relatively inefficient, but, nevertheless, a fraction with an approximately 60% limit was obtained from the barley starch. In the case of the malted-barley with a much higher percentage of small molecular size amylose, such a separation might well be expected to be even less efficient, but there was in fact the same trend in properties.

The nature of the barrier to the complete β-amylolysis of amylose is not yet known with certainty,[10] and will not be discussed here.

General pattern of change in starch during malting.—The effect of enzymic activity on the starch granule during malting is little understood, but some general pattern can be obtained from the above results.

The 16% loss in weight of starch during malting can be accounted for by the 28% loss in weight observed for the amylopectin component, if this is assumed to be degraded enzymatically to soluble products. Further, such a degradation of amylopectin would entail a corresponding increase in apparent amylose content to 25·5% in the malted-barley starch. This agrees with the experimental value of 26%. It would appear therefore that the degradation of amylopectin, to give soluble products, is more likely to account for the increase in amylose content than either of the possibilities of synthesis of more amylose, or the conversion of the exterior chains of amylopectin into linear material by some type of debranching enzyme. Enzymic degradation of part of the amylopectin to form soluble products would explain the initial loss in starch, the apparent increase in amylose content, and probably also the decrease in granular size on malting. This is in agreement with earlier work.[3,12]

During malting the granules could be subjected to attack by both α- and β-amylase. Microscopic examination of the malted-barley starch granules showed in fact evidence of amylolytic attack. A high percentage of the granules possessed the characteristics of the granules shown in the photomicrographs in the comprehensive review

TABLE IV

PROPERTIES OF AMYLOSE FRACTIONS

(Obtained by successive aqueous leaching of granules followed by dispersion of the residue)

Starch	Procedure (temperature, ° C.)	Amylose extracted (% of total)	Iodine affinity	Limiting viscosity no. [η]	Degree of polymerization	β-Amylolysis limit*
Barley ..	70°—leach of granules ..	26	19·0	89	660	(i) 96, (ii) 101
	80°—leach of residue ..	15	18·9	146	1,180	(i) 74, (ii) 100
	90°—leach of residue ..	20	18·2	246	1,800	(i) 65, (ii) 98
	Dispersion of residue ..	39	17·5	272	2,000	(i) 62, (ii) 95
Malted barley..	70°—leach of granules ..	15	19·0	66	490	(i) 97, (ii) 100
	80°—leach of residue ..	16	18·8	126	930	(i) 84, (ii) 100
	90°—leach or residue ..	30	18·6	212	1,560	(i) 74, (ii) 98
	Dispersion of residue ..	39	18·0	220	1,640	(i) 70, (ii) 98

* As Table II.

article by Sandstedt.[18] The granules still retained however their characteristic birefringent properties.

The separated components also show evidence of having been subjected to amylolytic attack. The observed increase in β-amylolysis limit for the malted-barley amylose can be accounted for simply by postulating α-amylolytic action on the original barley amylose. For example, the molecular size of amylose fraction 2 from the malted barley (see Table II) corresponds to 1·12 bonds broken per initial amylose molecule with a β-limit of 72%. If it is assumed that the barrier to β-amylolysis is randomly situated in the molecule, it can be calculated that the theoretical limit for 1 bond being broken in such a molecule is 86%. This is in good agreement with the observed limit of 90%. It must be stressed that the extent of α-amylolytic activity is very small. β-Amylolytic attack—which on amylose is of the "single-chain" type[10]—would have caused an apparent *lowering* in β-amylolysis limit and is therefore unlikely to have occurred. On this basis, therefore, there is no necessity to postulate the synthesis in malted-barley starch of amylose with properties different from those of the original barley amylose.

The amylopectin from the malted-barley had the same internal chain-length as the original barley amylopectin, and differed only in its external chain-length. This suggests limited β-amylolytic attack. No α-amylolytic attack would appear to have occurred, in view of the high molecular size as shown by the sedimentation behaviour.

It would appear therefore that, in the starch from malted barley, (*a*) the amylose has undergone limited α-amylolytic attack with virtually no β-amylolysis, and (*b*) the amylopectin has undergone limited β-amylolytic attack and no α-amylolytic attack. The limited enzymic attack during the time of flooring (*cf.* Sandstedt[18]) suggests that either there must be an amylase-inhibitor liberated during germination, or the granules are undamaged and not accessible to major enzymic attack. Sandstedt[18] has in fact shown that there is relatively little enzymic attack on undamaged granules and that degradation which does occur is on the *outside* of the granule. This observation may account for the preferential attack on amylopectin, as this component is thought to be associated primarily with the outside of the granule.[6] Further, the preferential attack on amylopectin is to be expected in view of the large number of non-reducing end-groups available in this molecule with a consequently high "turn-over" number.[4] The limited α-amylolytic attack on the amylose may be related to the fact that amylose is more accessible to the diffusion of reagents into the granule.[6] It would appear therefore, that properties of malted-barley starch can be explained on the basis of amylolytic attack on the original barley starch during malting rather than necessitating the degradation of the original granules and their resynthesis in a different form.

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The Fine-Structure of the Amylose Component of Starch *)

By W. Banks and C. T. Greenwood, Edinburgh/Scotland

With the exception of glutinous varieties, native starch granules are composed of at least two components - essentially linear amylose and highly branched amylopectin. The separated components bind iodine to different extents, and quantitative determinations of purity can be made from suitable potentiometric measurements (1). Separation of the components can be achieved by either simple aqueous leaching of the granule (2), or complete dispersion of the granules into aqueous solution and precipitation of the amylose as a complex with a polar organic molecule (2). Although examination of the purity of the residual amylopectin shows that dispersive methods give a more complete separation of amylose, the older method of aqueous leaching is perhaps more useful for fundamental studies of granular structure and the fine-structure of the components (3).

*) This is Part XIX in the Series "Physicochemical Studies on Starches".

Introduction

Early concepts of the chemical structure of amylose were, in fact, derived from a study of the products of aqueous leaching. Meyer and his co-workers (4)

leached maize starch at 80°. On methylation of the product and subsequent hydrolysis the yield of tetra-O-methyl glucose end-group corresponded to an average length of unit-chain of about 300 anhydroglucose units (5). The essentially linear nature of the molecule was then first established when the molecular weight of the acetylated derivative (by osmotic pressure measurements) corresponded to a degree of polymerisation (D. P.) of the same order (6). A similar result was found for potato amylose (7). Although from the results of similar experiments, HESS and his co-workers (8) claimed that potato amylose was branched to some extent, HASSID and MCCREADY (9) were able to confirm completely MEYER's results. However, the accuracy of such chemical methods is limited for the experimental percentage of end-group may be increased due to both probable drastic degrading effect of the methylation reagents, and the presence of contaminating amylopectin (in the early work there was in fact no method of measuring purity).

More recently the technique of periodate oxidation (10) has been applied to this problem. The method has the advantage that no derivative need be formed. The comparison of chain-lengths determined by this method with molecular weight values of the corresponding acetates has suggested that some amyloses may be branched (11). It is, however, again unlikely whether a chemical method is of much value when dealing with such long-chain molecules, particularly as more recent work has shown that the degree of polymerisation of undegraded amylose is more likely to be thousands of units rather than hundreds. A method based on the periodate oxidation of amylitol (the reduced form of amylose obtained by boron hydride reduction) has recently been proposed (12), but our own (unpublished) observations suggest that serious degradation occurs during the reduction process.

Further proof of the linear nature of the amylose molecule came from a study of the action of β-amylase. This enzyme will degrade amylose from the non-reducing end by the step-wise removal of maltose (13). A linear and unmodified chain of $1 \rightarrow 4$-α-linked anhydroglucose units is degraded completely. This was, in fact, found to be the case for MEYER's potato amylose. However, as will be seen below, there are difficulties in accepting without reservation even this conclusion.

The enzymic degradation of amylose

The idea of amylose as a simple unmodified straight-chain molecule persisted until 1949, when PEAT, WHELAN and PIRT (14) reported that crystalline sweet potato β-amylase (15) converted only about 80% of potato amylose into maltose[1]). These workers then studied soya-bean β-amylase (16), and showed that it could be fractionated to yield (i) a purified β-amylase which, like the crystalline enzyme, did not completely hydrolyse potato amylose, and (ii) another enzyme, Z-enzyme, which was capable of rendering the amylose susceptible to complete hydrolysis by the purified β-amylase. On this basis, therefore, amylose cannot be a simple unmodified chain of glucopyranose units linked by $1 \rightarrow 4$-α-D-glycosidic linkages.

This concept was vigorously criticized by MEYER (17), but the position is not simple. We have established that amylose itself is heterogeneous and can be separated into fractions, which are degraded by purified β-amylase to differing extents (3); the extent of β-amylolysis of any amylose thus depends on its method of preparation. There can be little doubt, however, of the presence of „Z-enzyme". But its specificity is open to doubt, as is the nature of the barrier to β-amylolysis.

β-amylase must be carefully purified and tested before use. It is relatively easy to destroy Z-enzyme by a suitable heat treatment (16), or to inactivate it by using a more acid medium for the digest (16). However, the former method is far more satisfactory than that using a mixture of β-amylase and Z-enzyme at a pH of 3.6. The crystallisation of β-amylase is difficult. BALL's first accomplished this for sweet potato (15), and later barley malt (18) and wheat (19) have been reported. (It should be noted that crystallisation in itself is far from a completely satisfactory criterion of purity (20).) We have attempted to crystallise soya-bean β-amylase, but have found this to be difficult in agreement with PEAT, WHELAN and PIRT's conclusions (16).

Fractionation and subfractionation of starch

Dispersive methods of fractionation give almost complete separation of amylose from amylopectin as shown by iodine titration experiments. The molecular size of the product, however, depends entirely on the methods used to isolate and purify the starch, and the careful minimization of oxidative degradation (21)

Table 1

Effect of Pretreatment of Starch and Variation in Fractionation Conditions on the Properties of Amyloses obtained by Complete Dispersion

Starch [1])	Pretreatment	Atmosphere during dispersion	D. P. [2])	β-limit [3])	Ref.
Potato (L1)	None	N_2	3200	77	22
	KOH	N_2	2900	—	22
Potato (L2)	None	N_2	2800	80	22
	Liquid NH_3	N_2	3000	76	24
Potato (L3)	None	N_2	3500	83	22
	None	O_2	1600	75	22
	None	Air	2600	79	22
Potato (C)	None	N_2	2500	78	22
Wheat (L)	None	N_2	*1950*	*65*	*24*
	KOH	N_2	*1900*	—	*24*
	Liquid NH_3	N_2	2100	68	*24*
Wheat (C)	None	N_2	980	—	23
Barley (L)	None	N_2	1800	72	24
	Liquid NH_3	N_2	1850	73	24

[1]) These authors quoted a β-amylolysis limit of 72%, but when the purity of their amylose is calculated from the limit reported for the action of crude soya-bean β-amylase on the sample, calculation shows that 79% of the amylose was actually converted to maltose.

[1]) L = Laboratory-prepared starch;
C = Commercial starch;
[2]) D. P. = Degree of polymerisation;
[3]) Expressed as percentage conversion into maltose.

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The Starch of the Tuber and Shoots of the Sprouting Potato

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(*Received* 18 *March* 1959)

Relatively few chemical investigations have been carried out on the amylose content of the potato-starch granule during growth (see Greenwood, 1956). Meyer & Heinrich (1942) isolated starch from the leaves, shoots and tubers and reported the amylose contents to be 22, 73 and 37 % respectively. The percentage of amylose determined by potentiometric iodine titration of the starch in the growing tuber was shown by Halsall, Hirst, Jones & Sansome (1948) to remain constant at 17 % for two varieties. These authors also found the average length of unit chain of the amylopectin components (calculated from periodate oxidation results on the whole starch) to be constant at 24–26 anhydroglucose units. There is, however, evidence that the percentage of amylose may vary with the botanical variety of the tuber. Anderson & Greenwood (1955) have found small variations in iodine affinity of

3·94–4·03 %, representing changes of 20–21 % of amylose, and Doremus, Crenshaw & Thurber (1951) have reported variations of 17·5–21·7 % in 22 varieties of sweet potato.

In the work reported here we have studied the physical and chemical characteristics of the starch isolated from the shoots and tubers of the sprouting potato in comparison with those of the original tuber starch.

EXPERIMENTAL

Isolation of starches. Starch was isolated immediately after harvesting the tubers (var. Home Guard) by extraction with 0·01 M-$HgCl_2$ (to inhibit enzymic activity), sedimentation of the granules after filtration and deproteinization by shaking with toluene and 4% NaCl soln. as previously described (Cowie & Greenwood, 1957*a*). Some tubers were then kept at room temperature in semi-darkness until sprouts of up to 3 in. in length were formed. These were removed and the starch in them was isolated as described above. The residual flaccid tubers were similarly extracted. Although no quantitative estimate was made, the yield of starch from all three sources was high.

Estimation of granular size distribution. Size distributions were obtained by enlarging photomicrographs (four or five fields per sample) to give a final magnification of about 350 diameters and by measuring with a rule (to the nearest millimetre) the size of at least 500 granules for each starch. In view of the ellipsoidal nature of potato-starch granules, each was characterized by the length of its major axis (d). Number-average and weight-average particle diameters were calculated from $\Sigma n_i d_i / \Sigma n_i$ and $\Sigma n_i d_i^4 / \Sigma n_i d_i^3$ respectively, where n_i is the number of granules of diameter d_i. (In the latter case, this assumes that the ratio major:minor axis was constant for all granules.)

Gelatinization temperatures. The number of granules gelatinized at a particular temperature in water was determined with a microscope and Kofler electrically-heated microscope stage as described by Schoch & Maywald (1956).

Determination of phosphorus. Percentages of phosphorus in the starches and their components were determined by wet oxidation of the polysaccharide with perchloric acid (Smith, 1953), followed by colorimetric estimation of the phosphomolybdate complex (Fogg & Wilkinson, 1958). Weights of polysaccharide taken were: amylose, 500 mg.; starch, 150 mg.; amylopectin, 100 mg. Results were reproducible to ±5% for the starch and amylopectin and to ±15% for the amylose.

Aqueous leaching and fractionation. Portions of the starches were leached with water at 60° for 10 min. under N_2 and the amylose was recovered as the butan-1-ol complex. Fractionation was achieved by complete dispersion of the starch in water by boiling for 1 hr. under N_2, followed by the addition of thymol to precipitate the amylose. The amylose was purified by reprecipitation as the butan-1-ol complex. These methods have been given in detail elsewhere (Cowie & Greenwood, 1957*b*).

Characterization of the fractionation products. The purity of the amylose and amylopectin components was obtained by potentiometric iodine titration with a semi-micro differential apparatus (Anderson & Greenwood, 1955). Titration conditions were: [I′], 0·01 M; [total free I_2]$_{max.}$, 10 μM; temperature, 20°. Limiting viscosity numbers [η] were obtained graphically from $[\eta] = \lim_{c \to 0} \eta_{sp.}/c$, where $\eta_{sp.}$ is the specific viscosity; concentrations (c) are expressed in g./ml. Measurements were made on the components dissolved in M-KOH at 22·5° with a modified Ubbelohde viscometer; detailed procedures are given by Cowie & Greenwood (1957*a*). A Spinco model E analytical ultracentrifuge was used to obtain the sedimentation constants (S_{20}) of the amylopectin components dissolved in water. The procedure and methods of evaluating the data have been described by Greenwood & Das Gupta (1958). Number-average degrees of polymerization for the amylose fractions were calculated from the relation: number-average degree of polymerization = 7·4 × [η] (Cowie & Greenwood, 1957*b*). The percentage conversion of samples of both amylose and amylopectin into maltose under the action of purified soya-bean β-amylase (Peat, Pirt & Whelan, 1952) was determined as described previously; the enzyme was free from maltase and α-amylase activity and contained no Z-enzyme (Bryce, Cowie, Greenwood & Jones, 1958). [Z-Enzyme (see Peat, Thomas & Whelan, 1952) is capable of effectively removing barriers to β-amylolysis found in amylose.] Average lengths of unit chain for the amylopectins were calculated from the results of a potentiometric estimation of the formic acid released on oxidation with $NaIO_4$ at 2° (Potter & Hassid, 1948).

RESULTS AND DISCUSSION

Physical characteristics of the starches

Potentiometric-titration experiments indicated small but reproducible differences between the iodine affinity of the different starches. Corresponding percentages of amylose are shown in Table 1. The starch from the shoots with 16·7 % of amylose differs significantly from the other two.

Table 1. *Physical properties of the starches*

Average gelatinization temperature is the temperature at which 50 % of the granules are gelatinized.

Starch source	Iodine affinity (mg. of I_2 bound/100 mg. of starch)	Amylose* (%)	Av. granular diam. (μ) No.	Av. granular diam. (μ) Wt.	Av. gelatinization temp.	Phosphorus (%)
Original tuber	3·94	20·2	42	55	61°	0·093
Shoots	3·25	16·7	14	19	61·5	0·071
Sprouted tuber	3·96	20·3	40	51	62·5	0·071

* Calculated from [(iodine affinity ÷ 19·5) × 100]; Cowie & Greenwood (1957*b*).

Fig. 1 shows the number- and weight-percentage distribution curves for the granules. Calculated averages are given in Table 1. The two tuber starches possess granules of similar size and differ only in that the proportion of large granules (i.e. $>80\,\mu$) is greater in the unsprouted tuber starch. This difference is emphasized in the weight-distribution curves. The size–distribution curves for the shoot starch are completely different, the average granular size being only about a third of that for both tuber starches. This suggests that the shoot starch may be immature.

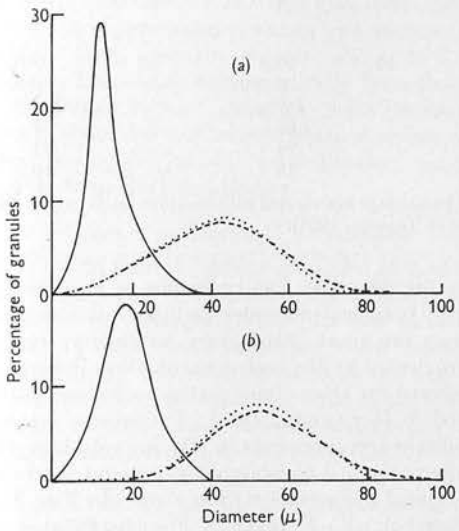

Fig. 1. Size–distribution curves for (*a*) number percentage and (*b*) weight percentage of granules. – – –, Starch from original tubers; —, starch from shoots; ..., starch from sprouted tubers.

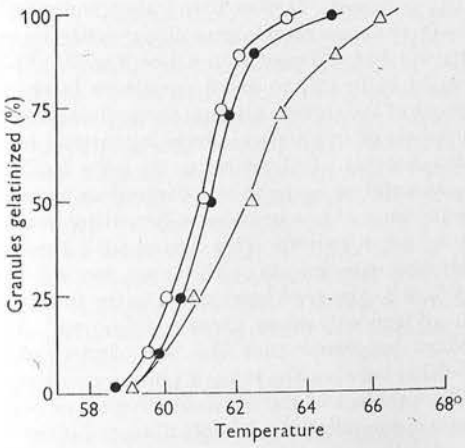

Fig. 2. Percentage of granules gelatinized in water as a function of temperature. ○, Starch from original tubers; ●, starch from shoots; △, starch from sprouted tubers.

Gelatinization curves are shown in Fig. 2, and the average gelatinization temperatures are given in Table 1. The curves are typical and illustrate the range of about 5° over which gelatinization of a starch occurs. The differences observed between the starches are again reproducible but are small and not of obvious significance. Granule size, the percentage of amylose present, the molecular weight of the components and the corresponding micellar organization of the granule may all influence gelatinization. An increase in amylose content raises the average gelatinization temperature (Savage, Deatherage, MacMasters & Senti, 1958). On the other hand, granular size is also important, as in any experiment large granules are seen to gelatinize first, whereas a few small ungelatinized granules often remain when the process is otherwise complete. For the two tuber starches, which have the same amount of amylose, the effect of the very large granules in the original starch may account for the differences in average gelatinization temperature. Although the shoot starch contains less amylose, the granular size is small. The observed gelatinization temperature must be accounted for by the interplay of these two effects, although the whole phenomenon is very complex.

The phosphate content of the original tuber starch appears to be higher than that for the other two starches. [Phosphorus occurs in potato starch as phosphate on C_6 of the glucose unit (Posternak, 1950).]

Aqueous leaching and fractionation

Our previous work (Cowie & Greenwood, 1957*b*) has shown that when potato starch of different botanical varieties is dispersed and fractionated under careful oxygen-free conditions, an amylose with a degree of polymerization of 3000–4000 is obtained. Further, aqueous leaching at 60–70° results in the preferential extraction of an amylose fraction of relatively low number-average degree of polymerization (~ 2000). Table 2 shows that the original starch gave results in agreement with this, which supports the suggestion of Cowie & Greenwood (1957*b*) that the molecular size of amylose is to some extent independent of the botanical variety of potato. Essentially similar results were obtained for the starch from the sprouted tuber. Although these tubers had obviously undergone great changes in physical state, surprisingly little enzymic modification or degradation of the granular starch has occurred. However, the number-average degree of polymerization of the aqueous-leached and total amyloses from the shoot starch are both significantly smaller, whereas the percentage conversion of the total amylose into

Table 2. *Properties of the fractions obtained from aqueous leaching and dispersion of the starches*

Granules were leached with water at 60° for 10 min., or dispersed in water at 100° for 1 hr.; both experiments were carried out in N_2.

	Original tuber		Shoot		Sprouted tuber	
	Leach	Dispersion	Leach	Dispersion	Leach	Dispersion
Amylose						
Percentage of total amylose*	45	>95	45	>95	40	>95
Purity (%)*	>98	>98	—	>98	—	>98
Phosphorus (%)	—	0·005	—	0·001	—	0·004
$[\eta]$ (g./ml.)	270	450	210	315	270	435
Degree of polymerization	2000	3300	1600	2300	2000	3200
β-Limit†	—	83	—	94	—	83
Amylopectin						
Purity (%)*	89	99·5	89	99·5	88	99·0
Phosphorus (%)	—	0·111	—	0·089	—	0·097
$[\eta]$ (g./ml.)	—	160	—	160	—	160
β-Limit†	—	56	—	56	—	57
Chain length	—	24	—	24	—	24
External chain length‡	—	16	—	16	—	16

* Calculated from iodine titrations. † Percentage conversion into maltose on β-amylolysis.
‡ Calculated from [(chain length × β-limit) + 2·5] (Peat, Whelan & Thomas, 1952).

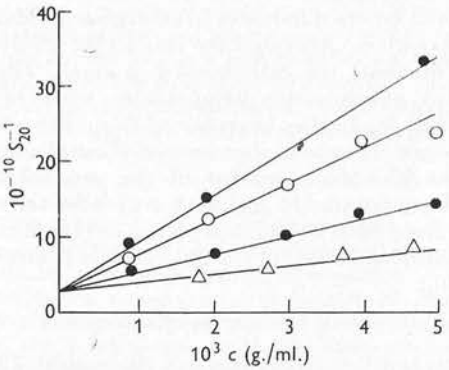

Fig. 3. Relation between the inverse of the sedimentation coefficient (S_{20}^{-1}) and the concentration (c) for the amylopectins dissolved in water. ○, Amylopectin from original tuber starch; ●, independent runs on amylopectin from shoot starch (see text); △, amylopectin from sprouted-tuber starch.

maltose on β-amylolysis is very much higher. The amount of phosphorus in this amylose was significantly lower.

On dispersion and fractionation the amylopectins obtained from all the starches were identical within experimental error in phosphorus content, average length of unit chain, percentage conversion into maltose with β-amylase and calculated average exterior chain lengths (see Table 2). As far as could be determined there was also little difference in the molecular size. Measurements of the molecular size of amylopectin are difficult (see Greenwood, 1956). Although the limiting viscosity numbers were identical, this is not conclusive as viscosity measurements are not particularly sensitive for detecting small changes in size for such highly branched molecules. Sedimentation measurements are more satisfactory in theory, but are complicated by the high concentration-dependence observed for the sedimentation coefficient (Greenwood & Das Gupta, 1958). Previously, we have made the measurements in alkaline solution. Here we have studied aqueous solutions, and the results of typical measurements are shown in Fig. 3. In agreement with Erlander & French (1958) it was found that the graph S_{20}^{-1} versus c (where S_{20} is the sedimentation coefficient corrected to water at 20°) was linear and allowed extrapolation to infinite dilution. However, the accuracy of such an extrapolation is limited in view of the high values of S_{20} involved. It was found that independent runs on the same sample gave curves with the same intercept but different slopes (see Fig. 3). This is thought to be due to small variations in the salt content of the solvent altering the ionization of the phosphate groups and so influencing intermolecular entanglements. Sedimentation in 0·2 M-NaCl was unsuccessful as aggregation occurred as shown by the presence of two or more sedimenting peaks on the schlieren pattern. This agrees with the light-scattering experiments of Witnauer, Senti & Stern (1955), who observed aggregation in the presence of salt of concentrations greater than mM. These authors suggested that the salt destroyed the repulsion between the ionized phosphate groups.

The problem of the ultracentrifuging of amylopectin is complicated, and only a limited number of investigations have yet appeared in the literature (Greenwood & Das Gupta, 1958; Erlander & French, 1958; Stacy & Foster, 1957). Further studies are being made. At most, it can be said

that S_{20} for the potato amylopectins studied here in aqueous solution is large and probably at least 500×10^{-13} c.g.s. units.

Utilization of the starch granule

The similarity in physical and chemical properties of both tuber starches suggests that when granules are utilized enzymically they are individually completely broken down to soluble material rather than being partially attacked. Furthermore, the extremely large granules may well be utilized first, a fact which may be related to their looser organization as shown by gelatinization studies. Both starches contain about 20% of amylose. This value differs radically from that of 37% found by Meyer & Heinrich (1942). However, these authors did not isolate whole granules and their procedure would preferentially extract amylose from the plant tissues.

Nature of the barrier to β-amylolysis

The starch from the shoots contained 16% of the linear component, in contrast to the 73% reported by Meyer & Heinrich (1942). As the starch is unlikely to have been transported to the shoots in granular form, the possibility exists that it may be immature. Also, the molecular size of the corresponding amylose is considerably smaller than for the tuber starches and the barrier to β-amylolysis (see, Peat, Pirt & Whelan, 1952) is more limited. The nature of the barrier to β-amylolysis is uncertain. It may be an artifact introduced during the isolation of fractionation of the starch (see, Baum, Gilbert & Scott, 1956). Equally well it may be native to the amylose. In the latter case the possibility of phosphorus giving rise to the barrier must be considered (compare, however, Peat, Thomas & Whelan, 1952). The amount of phosphorus in all the amyloses is very small (see Table 2) and consequently its estimation is difficult. Also, the presence of an amylopectin impurity of about 1% would cause a considerable error in the given phosphorus contents. Nevertheless, it is thought significant that the shoot amylose, which has the highest conversion into maltose on β-amylolysis, should have the lowest phosphate content. The problem is, however, highly complex and is unlikely to be solved until the specificity of Z-enzyme is finally elucidated [cf. Peat, Thomas & Whelan (1952), Hopkins & Bird (1953) and Neufeld & Hassid (1955)].

SUMMARY

1. Starch has been isolated from the shoots and tubers of sprouting potatoes, and its physical and chemical properties have been compared with those of the starch from the original tubers.

2. Although the two tuber starches were similar, that from the shoot was profoundly different and possessed a lower iodine affinity and smaller granular size.

3. The amylose fractions separated from the different starches varied both with regard to molecular size and β-amylolysis limit, whereas the amylopectin fractions appeared to be similar.

4. The sedimentation of amylopectin has been studied.

5. The nature of the barrier to β-amylolysis is discussed.

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693. *Physicochemical Studies on Starches. Part XX.* The Existence of an Anomalous Amylopectin in Starch.*

By W. Banks and C. T. Greenwood.

The polysaccharide from the supernatant liquor from the recrystallisation of the initial amylose-complex of potato starch has a significantly lower average chain length and β-amylolysis limit than amylopectin. A similar polysaccharide occurs in rubber-seed starch. This material, which represents 5—10% of the granule, is thought to be inherent in the granular structure. Its physical properties are discussed.

Although the dual-component nature of the starch granule is well established,[1] there are reports of the existence of material with properties different from those of amylose and amylopectin. From a comprehensive study of the iodine affinities of various fractions and subfractions of amylose, Schoch and his co-workers [2] have suggested that in maize starch there may be 5—7% of such a substance. This polysaccharide was apparently precipitated by "Pentasol" but not by butan-1-ol. On the basis of optical-density and β-amylolysis measurements on mixtures of pure potato amylose and amylopectin, Peat, Pirt, and Whelan [3] postulated that potato starch granules may contain material with a higher iodine affinity but a lower β-amylolysis limit than amylopectin. An anomalous "thymol-amylopectin" has been isolated from the supernatant liquors from the recrystallisation of the initial thymol-amylose complex of potato starch by Cowie and Greenwood.[4] Recently, Perlin [5] has isolated a similar fraction (termed "Amylopectin-C") from wheat starch, whilst a second component, of low molecular weight, in waxy maize starch has been reported from sedimentation studies by Erlander and French.[6] In this paper, we give the results of detailed investigations made on the "thymol-amylopectin" isolated from potato starch and rubber-seed starch.

Experimental

Methods of isolating potato starch (vars.: Epicure = potato 1; Redskin = potato 2; Golden wonder = potato 3) and fractionating it so as to obtain the "thymol-amylopectin" have been described previously.[4] The polysaccharide was isolated by freeze-drying the supernatant liquor which had been concentrated to small volume under reduced pressure (temp. $<35°$). For Epicure starch, fraction 1A was isolated in this way, whilst fraction 1B was obtained by freeze-drying the supernatant directly. All samples were exhaustively extracted with boiling methanol. The sample of polysaccharide from rubber-seed starch had been isolated in earlier work.[7] Samples were characterised by measurements of iodine affinity (I.A.), average length of unit-chain ($\overline{C.L.}$), limiting viscosity number $[\eta]$, and percentage conversion to maltose on β-amylolysis (see earlier papers in this series [7]). The percentage of phosphorus in some samples was determined by wet oxidation of the polysaccharide with perchloric acid,[8] followed by colorimetric estimation of the phosphomolybdate complex as described by Fogg and Wilkinson.[9] (Sample weights of *ca.* 250 mg. were taken, and results were reproducible to $\pm 5\%$.)

Subfractionation of "thymol-amylopectin." A 0·25% aqueous solution of the Epicure potato "thymol-amylopectin" (250 ml. containing 0·1% of sodium chloride) was kept at 30°. Ethanol was added slowly with vigorous mechanical stirring until a permanent turbidity was observed (some 20% by volume was required). The temperature was then raised until the turbidity disappeared (50—55°), and the flask was then allowed to cool to 30° during 6 hr. After a further 48 hr. at this temperature, the supernatant liquor was decanted from the precipitated gel. A second fraction was precipitated by raising the alcohol concentration to 40%. Both fractions were dispersed in water and freeze-dried to yield fractions 1C and 1D, respectively. (Each fraction contained *ca.* 50% of the original polysaccharide.)

* Part XIX, *Stärke*, in the press.

Results and Discussion

The results shown in the Table indicate that about 5—10% of potato starch appears to be precipitated with the initial thymol-amylose complex, and can be isolated from the recrystallisation liquors. A similar result has been found with butan-1-ol as the initial precipitant, but other reagents have not been investigated. This polysaccharide has a low iodine affinity, but a significantly lower chain length and β-amylolysis limit than amylopectin. The material would normally be discarded during fractionation.

Properties of amylopectin-type polysaccharides in starches.

Starch	Component	Amount (%) [a]	Amylose (%) [b]	$\overline{C.L.}$	β-Limit [c]	External chain length [d]	[η]
Potato	Amylopectin 1	~75	0·5	23·1	57	15·7	190
	" Thymol-amylopectin " 1A	5—10	1·0	13·4	53	9·6	170
	" " 1B	5—10	0·5	13·6	51	9·4	150
	" " 1C	—	—	13·8	52	9·7	—
	" " 1D	—	—	14·0	50	9·5	—
	Amylopectin 2	~75	0·5	23·3	57	15·9	200
	" Thymol-amylopectin " 2	5—10	5·0	14·7	53	9·3	220
	Amylopectin 3	~75	1·0	23·2 *	56	15·5	180
	" Thymol-amylopectin " 3	5—10	2·0	15·8 *	—	—	76
Rubber-seed	Amylopectin	~70	0·5	23·1 *	64	17·3	—
	" Thymol-amylopectin "	10	2	15·8 *	61	12·1	—
Wheat †	Amylopectin	~70	low	Not accurately measured	55—60	—	—
	" Amylopectin C "	5—10	low		48—53	—	—

[a] % of total starch. [b] Calc. from iodine affinity. [c] Expressed as percentage conversion into maltose. [d] Calc. from $\{[\overline{C.L.} \times (\beta\text{-limit})] + 2{\cdot}5\}$.

* Results by courtesy of Mr. J. Thomson. † Results from ref. 5.

The values of chain length and β-amylolysis limit for the " thymol-amylopectin " from rubber-seed starch and the " Amylopectin-C " from wheat starch are comparable to those for the potato polysaccharide. It is therefore suggested that such a polysaccharide might be a universal constituent of starches.

Adsorption of this branched polysaccharide on the initial amylose complex is unlikely, for the iodine affinity of the complex was unaltered by repeated washing with thymol-saturated water, or varying the force-field used in the isolation, or using butan-1-ol instead of thymol. Further, on dispersion of a starch, which had been previously leached with water at 65° and had lost about 60% of its amylose, the resultant amylose–thymol complex was 45% pure. If it is assumed that the same amount of branched polysaccharide is co-precipitated, the calculated purity of the complex is *ca.* 50%. This agrees well with the observed value. It is not known why co-precipitation should occur. There was the possibility that the phosphate groups in potato starch were predominantly associated with this fraction, but the " thymol-amylopectin " from Epicure starch contained 0·07% of phosphorus, whilst there was a similar amount (0·09%) in the corresponding amylopectin. Phosphate was therefore unlikely to be causing co-precipitation. Further, rubber seed and wheat starch have little phosphate. Although the purity of the thymol-amylose complex was independent of the length of time of dispersion of the starch for up to 2 hr. (*i.e.* the usual conditions for fractionation), the polysaccharide could be residual undispersed granular particles. These might disperse in the more dilute conditions of the recrystallisation. This will be discussed further below.

Although a component with properties intermediate between those of amylose and amylopectin (*e.g.*, an amylopectin with external chains of sufficient length to form complexes with thymol and other reagents) might be expected,[1] " thymol-amylopectin " is not such a molecule. On the basis of β-amylolysis limits, the external chain length is only 9—11 anhydroglucose units compared with 15—16 in amylopectin. Erlander [10] has suggested that glycogen may be a precursor for the synthesis *in vivo* of amylopectin, but although

the chain length of the polysaccharide approaches that for glycogen, its physical behaviour is inconsistent with this concept. (It is to be noted that on subfractionation of the polysaccharide, fractions 1C and 1D showed no variation in chain length and β-limit, which may indicate that the polysaccharide is not a mixture of amylopectin and glycogen.) The limiting viscosity number of *ca.* 180 is little different from that for amylopectin (whereas the value for glycogen [11] is *ca.* 10). The relatively low limiting viscosity number suggests that a molecule of "herring-bone" type of structure (*i.e.*, a main chain with side chains of *ca.* 15 glucose units) is unlikely. Rather would the polysaccharide appear to be degraded amylopectin. It is not thought that the difference in internal chain lengths of 5—6 glucose units for the polysaccharide compared with 7—8 units for amylopectin is significant.

The molecular weight of the "thymol-amylopectin" cannot be accurately determined. Ultracentrifugal examination showed that the properties of both the initial amylose-thymol complex and the corresponding "thymol-amylopectin" varied with the variety of potato starch. The initial thymol complex was not always homogeneous (cf. ref. 4). The isolated "thymol-amylopectin" showed a small but variable amount of rapidly sedimenting material—probably amylopectin; estimates of the amount of the latter are made difficult by the Johnston–Ogston effect.[12] However, unpublished light-scattering experiments by Mr. I. G. Jones have indicated that the dissymmetry and the molecular weight (*ca.* 10^8) of "thymol-amylopectin" were of the same order as those of the parent amylopectin.

It is suggested that "thymol-amylopectin" could be amylopectin which has undergone enzymic modification—either by premature cessation of synthesis, or by degradation. As amylopectin is mainly on the outside of the granule,[13] any enzymically-modified material might be associated with residual "granular sacs." The material may be therefore an inherent part of the granule, but the amount and the molecular properties will depend entirely on the botanical environment of the source of the starch.

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29. *Physicochemical Studies on Starches. Part XXI.* Observations on Z-Enzyme.*

By W. Banks, C. T. Greenwood, and I. G. Jones.

The action pattern of the Z-enzyme associated with preparations of β-amylase has been critically studied by viscosity and light scattering. Solutions of the enzyme were obtained by selective inhibition of the β-amylase with mercuric chloride. The enzyme degraded, in a manner consistent with a random hydrolytic action, both linear amylose and amylose containing a barrier to β-amylase. Limited hydrolysis of amylopectin β-limit dextrin occurred, whilst there was no action on the β-limit dextrin of glycogen. The hydrolytic action of Z-enzyme on amylose and amylopectin is indistinguishable from that of an α-amylase. The implication of these findings regarding the nature of the barrier to β-amylase in amylose is discussed.

The nature of the barrier to the complete β-amylolysis of amylose is not yet known. We have shown, however, that the barriers may be due to an artefact as they can be introduced by treating a heated solution of amylose with oxygen.[1] Such artificially induced barriers can be effectively removed [2]—as can those which normally occur in amylose—by the Z-enzyme described by Peat, Pirt, and Whelan.[3] These results suggested that Z-enzyme was either specific for the removal of oxidised anhydroglucose residues or had a random hydrolytic action. In a effort to characterise amylose completely, as a preliminary to detailed studies of hydrodynamic behaviour, we have now investigated the action of Z-enzyme. The nature of this enzyme has been in dispute.[3–7] Peat, Pirt, and Whelan [3] have shown that it is heat-labile, is inactivated at pH 3·6, and has optimum activity about pH 6 at which pH the barriers to β-amylase present in amylose are effectively removed. The original evidence [4] that Z-enzyme had no α-amylolytic activity but was a β-glucosidase has been questioned.[5,6] As we were unable to repeat the isolation [4] of Z-enzyme from impure preparations of soya-bean β-amylase, we have employed *selective inhibition* of β-amylase in samples containing both enzymes. This technique has proved to be both exceptionally simple and effective. Z-Enzyme was present in only small amounts, therefore physicochemical methods were essential to study the enzyme action pattern; chemical methods dependent on measurements of reducing power and iodine-stain were insensitive to detect the limited changes involved.

Experimental

Isolation of Substrates.—Amylose samples I and II were isolated from dispersions of potato starch (vars. Epicure and Redskin) as described earlier.[8] Amylose IIa was a linear subfraction of potato amylose obtained by aqueous leaching.[9] Amylose III was leached at 85° from oat starch (var. Milford). Amylose IV was a composite sample of subfractions of wheat amylose (var. Victor II).[9] Amylopectin was prepared from potato starch (var. Redskin).[8] Glycogen was isolated from brewers' yeast.[10]

Enzyme Preparations.—Purified and crude soya-bean β-amylase were prepared as described by Peat *et al.*[3,11] Commercial samples A, B, and C were obtained from Nutritional Biochemicals Corporation, Lights Ltd., and Wallerstein Laboratories Inc., respectively. (Sample C was kindly provided by Dr. Manners.) Maltase activity was negligible in all preparations. The percentage conversion into maltose of amylose I and potato amylopectin is shown in Table 1

* Part XX, *J.*, 1959, .

for these enzymes in digests of various values of pH, using *ca.* 50 units of β-amylase [12] per mg. of substrate.

Effect of Various Inhibitors on Enzymic Activity.—Digests were prepared with crude soya-bean β-amylase (50 units [12] of enzyme/mg. of amylose; pH 4·6) in the presence of the inhibitors shown below. After 48 hr. at 35°, the residual polysaccharide was isolated as the butan-1-ol complex and its β-amylolysis limit determined with purified soya-bean β-amylase. The results were as shown.

	β-Amylolysis limit [a]		Inhibition [b] of	
Inhibitor	original	final	β-amylase	Z-enzyme
Formamide (30% aq.; v/v)	82	83	+	+
Formamide (40% aq., v/v)	82	80	+	+
Ascorbic acid (1×10^{-2}M)	82	84	+	+
Mercuric chloride ($1{\cdot}5 \times 10^{-6}$M)	82	89	+	−

[a] Expressed as percentage conversion into maltose. [b] + = Complete inhibition; − = non inhibition.

Experiments involving Inhibition by Mercuric Chloride.—Digests were prepared as follows: enzyme solution (10 ml.; 2500 units of β-amylase [12]) and 0·2M-acetate buffer (10 ml.) were diluted to *ca.* 60 ml. with water, and $1{\cdot}5 \times 10^{-5}$M-mercuric chloride (10 ml.) was added. Amylose solution (10 ml.; *ca.* 100 mg. of amylose) was then added, and the whole diluted to 100 ml. and incubated at 37°; toluene was added to prevent bacterial action. Control experiments without enzyme were carried out similarly. After 48 hr. samples were withdrawn for estimation of reducing power,[9] and then the polysaccharide was precipitated as a complex with butan-1-ol. A small, variable amount (<5%) of amylose which retrograded from solution during this time was removed by centrifugation before the addition of alcohol.

Characterisation of Amylose.—The limiting viscosity number was determined on the butan-1-ol complex.[8] β-Amylolysis limits after treatment with Z-enzyme were obtained with purified soya-bean β-amylase at pH 3·6; details of this procedure have been given elsewhere.[9]

Light Scattering.—Amylopectin β-limit dextrin (*ca.* 10 mg.) was dissolved in water (25 ml.) and clarified by filtration under gravity through a G4 sintered-glass filter.[13] Enzyme powder (*ca.* 100 units of β-amylase/mg. of substrate) was dissolved in 0·2M-acetate buffer (3 ml., of appropriate pH), any undissolved material being removed by filtration. (This procedure avoided an immediate increase in turbidity observed when the enzyme was brought to an acid pH.) The enzyme solution was then added to the solution of the dextrin, and measurements was immediately made of the light scattered at 90° $[(R_{90})_0]$, a Brice–Phœnix photometer being used.[13] Measurements were then made after incubation for varying times to give $(R_{90})_t$.

Similar experiments were carried out on glycogen β-limit dextrin, the only difference being that the solutions were rather more concentrated (*ca.* 18 mg./25 ml.).

Results were expressed as $(R_{90})_t/(R_{90})_0$, and this ratio was plotted as a function of time (t) (see Figure). Variations in angular scattering were neglected and hence *absolute* values of molecular weights are not available, but changes in the Rayleigh ratio indicate changes in molecular weight, *i.e.*, any decrease in $(R_{90})_t/(R_{90})_0$ must represent a decrease in molecular weight of the polysaccharide, particularly as this ratio is liable to cause underestimation of any changes involved.

Z-Enzyme Activity.—The measurement of Z-enzyme activity is difficult. A relative measure was adopted in this work. Digests containing amylose, enzyme, and mercuric chloride at pH 5·5 were incubated for 1 hr. at 37°. After denaturation of the Z-enzyme by heat, the amylose was isolated as the butan-1-ol complex. The resultant decrease in limiting viscosity number of the amylose was taken as a measure of Z-enzyme activity. The results quoted below are the relative activities of Z-enzyme for equal amounts of β-amylase activity:

Sample	Commercial A	Commercial B	Commercial C	Crude soya-bean
Relative activity	1	2	3	6

Results and Discussion

The β-amylolysis results in Table 1 show that all the enzyme preparations, with the exception of purified soya-bean β-amylase, contained Z-enzyme. The crude soya-bean preparation apparently contained α-amylase as shown by an increase in the β-amylolysis limit for amylopectin at pH 5·5. A preparation from another batch of soya-beans behaved

similarly. However, this enzyme did not attack glycogen β-limit dextrin, and hence was not an α-amylase (see below).

The nature of the essential thiol groups in β-amylase was first established by Weill and Caldwell.[14] These authors[15] also showed that the enzyme could be inhibited by iodine, hydrogen sulphide, iodomercuric compounds, and iodoacetamide. More recently, non-competitive inhibition by ascorbic acid[16] has been studied. Other inhibitors which have been suggested include formamide[17] and mercuric chloride.[3] However, no reports

TABLE 1. *Properties of β-amylase samples.*

	β-Amylolysis limit [a] for					
	Amylose			Amylopectin		
pH of digest	3·6	4·6	5·5	3·6	4·6	5·5
Purified soya-bean	80	82	83	56	57	57
Crude soya-bean	83	98	99	55	57	63
Commercial enzyme A	81	98	100	56	56	57
Commercial enzyme B	83	100	99	57	56	57
Commercial enzyme C	82	100	100	56	57	57

[a] Expressed as percentage conversion into maltose.

of the inhibition of β-amylase in the presence of Z-enzyme appear to have been made. We therefore studied the effect of some of the above inhibitors on a mixture of β-amylase and Z-enzyme to see whether a preferential inhibition of β-amylase could be achieved. This was found to occur with mercuric chloride; the inhibition of β-amylase was complete, but Z-enzyme was unaffected (cf. ref. 4). This inhibitor was therefore used in all subsequent work to provide solutions of Z-enzyme.

The Action of Z-Enzyme on Amylose.—Table 2 shows the properties of various amyloses after treatment with Z-enzyme at pH's of 3·6, 4·6, and 5·5. The control experiments—in which amyloses were incubated without enzyme and also with inhibited purified β-amylase—show that the physical conditions caused no fundamental change in either β-amylolysis limit or limiting viscosity number. However, with the exception of those at pH 3·6, in all digests containing Z-enzyme, the limiting viscosity numbers of the isolated amyloses were considerably smaller than of the controls, whilst the β-amylolysis limits tended to 100% conversion. At pH 3·6, where Z-enzyme is inhibited, the properties of the treated and control amylose were comparable.

The decrease in viscosity was largest in those digests buffered to pH 5·5, in which region Z-enzyme has maximum activity (cf. ref. 4). Even at this pH, however, reducing sugars could not be detected in the digests by the alkaline ferricyanide reagent. When the enzyme : substrate ratio was substantially increased, by incubating *ca.* 20 mg. of amylose with 3,000 units[12] of commercial enzyme B in the presence of inhibitor, reducing sugars were still not apparent; the residual polysaccharide could not be precipitated with butan-1-ol, indicating considerable hydrolysis, but it still gave a typical blue stain with iodine.

It is difficult to postulate any arrangement of barriers in amylose such that a *specific* attack by Z-enzyme, at the site of these barriers, could result in the large decreases in viscosity reported in Table 2. The non-specific nature of Z-enzyme is further emphasised by its ability to attack sample IIa, a linear amylose, *i.e.*, one which is completely degraded by *pure* β-amylase and cannot therefore contain any barriers.

The large decrease in viscosity suggests, in fact, that random hydrolytic action of an α-amylolytic type is occurring. This will necessarily result in an increase in the β-amylolysis limit. If it is assumed that random degradation of amylose chains—having not more than one barrier per molecule—is occurring, then

$$L = 100 - \{(100 - L_0)[\eta]/[\eta]_0\}$$

where L_0 and $[\eta]_0$ are the original β-amylolysis limit and limiting viscosity number, respectively, and L and $[\eta]$ are the corresponding values for the degraded amylose. This

derivation assumes that $[\eta]$ varies as the molecular weight, which has been shown for our experimental conditions. Values for such theoretical β-amylolysis limits for the amylose

TABLE 2. *The action of Z-enzyme on amylose.**

Amylose	pH	$[\eta]_0$ †	$[\eta]$ †	L_0 ‡	L ‡	$L_{calc.}$ ‡	Amylose	pH	$[\eta]_0$ †	$[\eta]$ †	L_0 ‡	L ‡	$L_{calc.}$ ‡
Purified soya-bean							Commercial enzyme B						
II	3·6	440	430	83	82	83	II	3·6	440	440	83	84	83
	4·6	430	420	84	83	83		4·6	430	110	84	97	96
	5·5	420	435	84	83	83		5·5	420	30	84	100	99
Crude soya-bean							IV	3·6	190	190	71	72	71
I	3·6	360	370	81	81	81		4·6	195	80	74	92	89
	4·6	370	60	83	96	97		5·5	195	30	73	96	96
	5·5	370	20	83	100	99	Commercial enzyme C						
III	3·6	160	165	74	74	74	II	3·6	440	430	83	84	83
	4·6	170	75	74	88	89		4·6	430	145	84	95	95
IV	3·6	190	185	71	73	71		5·5	420	45	84	99	98
	4·6	195	80	74	93	89	IV	3·6	190	185	71	72	71
	5·5	195	25	73	98	97		4·6	195	115	74	85	85
Commercial enzyme A								5·5	195	45	73	94	94
II	3·6	440	440	83	83	83							
	4·6	430	290	84	89	89							
	5·5	420	120	84	94	95							
IV	3·6	190	190	71	73	71							
	4·6	195	160	74	77	79							
	5·5	195	80	73	87	89							
IIa	4·6	250	150	98	99	99							

* For digest conditions see text.

† Limiting viscosity number of amylose in control solution $[\eta]_0$, and amylose after treatment with Z-enzyme $[\eta]$.

‡ β-Amylolysis limit of amylose in control solution (L_0), after treatment with Z-enzyme (L), and calculated value, random degradation being assumed ($L_{calc.}$) [see text].

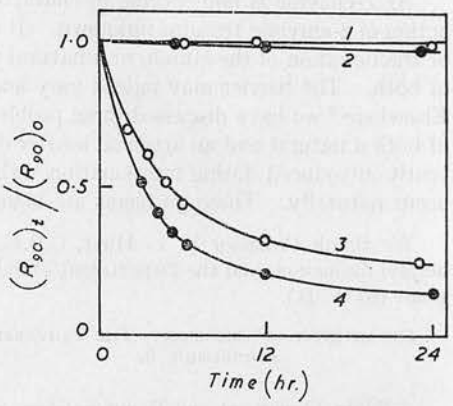
samples treated with Z-enzyme show excellent agreement with the observed values (see Table 2), thus again suggesting that the action of Z-enzyme on amylose is of an α-amylolytic type. On this basis, β-amylolysis of the amylose samples treated with Z-enzyme is not necessarily expected to be complete; the extent will depend simply on the concentration of enzyme (cf. activity measurements quoted in the Experimental section) and the pH of the digest.

The difference between the concurrent action of the two enzymes and the effect of β-amylase following Z-enzyme must be related to the high turnover number of the β-amylase.[18] Amylose is thought to consist [9] of a mixture of (i) completely linear molecules and (ii) those containing a randomly situated barrier; the percentage of the latter being *ca.* 45% for potato starch. When both enzymes are present, the β-amylase will rapidly degrade all the linear molecules completely and those with a barrier to *ca.* 50%. Further β-amylolysis will then occur as these resistant portions are concurrently hydrolysed by Z-enzyme. As the effective concentration of the resistant material will rapidly decrease, β-amylolysis of the amylose will soon be effectively complete. However, when Z-enzyme acts alone, it will hydrolyse a random selection of the amylose molecules present. Hydrolysis of a linear molecule will cause no change in the observed β-limit, whilst hydrolysis of a molecule with a barrier will cause an increase in subsequent β-amylolysis; the conversion will depend simply on the extent of hydrolysis.

The Action of Z-Enzyme on Amylopectin and Glycogen.—If Z-enzyme is an α-amylase, it should attack amylopectin and glycogen even though the extent of such reaction under our conditions will necessarily be very small. Previous claims [3] that Z-enzyme had *no* effect on these substrates were based on evidence of reducing power and iodine-staining measurements. However, neither of these methods is sensitive to limited degradation, and some physical technique for measuring changes in molecular size is required. The light-scattering method is the most convenient and accurate of these for following con-

tinuous changes and we have used it here. Typical results of such studies are shown in the Figure.

It is obvious that, whereas the amylopectin β-limit dextrin is degraded at those pH values at which Z-enzyme is active, the glycogen β-limit dextrin is unaffected. Furthermore, the enzyme present in the crude soya-bean preparation which degraded amylopectin at pH 5·5 (see Table 1) had also no effect on the molecular size of glycogen. This suggests that true α-amylase is absent, the increase in hydrolysis being brought about by the Z-enzyme. As the crude soya-bean β-amylase contained much more Z-enzyme than any other preparation (see Experimental section) a measurable increase in β-amylolysis limit would be expected when amylopectin is incubated with high concentrations of commercial enzyme. This was, in fact, found to be the case.



Action of Z-enzyme on amylopectin and glycogen β-limit dextrins as shown by light scattering.

Variation of $(R_{90})_t/(R_{90})_0$ [see text] as a function of time of incubation with Z-enzyme for (1) glycogen β-limit dextrin at pH 5·5, (2) amylopectin β-limit dextrin at pH 3·6, (3) amylopectin β-limit dextrin at pH 4·6, (4) amylopectin β-limit dextrin at pH 5·5.

Earlier Observations on the α-Amylolytic Nature of Z-Enzyme.—Peat *et al.*[4] suggested that Z-enzyme could not be a weak α-amylase because (1) α-amylases are inhibited by mercuric ions, whereas Z-enzyme is not, and (2) when Z-enzyme was replaced by salivary α-amylase, the β-amylolysis limit of the amylose did not reach 100% conversion. Hopkins and Bird [5] have already pointed out that the first argument is fallacious (cf. ref. 19). Further, our results suggest that comparison of Z-enzyme with a true α-amylase is impossible.

In an earlier paper,[20] we found that the mean sedimentation coefficient of amylose did not change appreciably during the action of β-amylase alone, or under the concurrent action of β-amylase and Z-enzyme. It was therefore concluded that not only did β-amylase degrade by a single chain mechanism, but that the constancy of molecular weight during degradation in the presence of Z-enzyme suggested that random degradation was not occurring. However, measurement of the change in mean sedimentation coefficient is not sufficient to detect changes in molecular-weight distribution. The mean value measures, in fact, only the sedimentation coefficient of the most abundant species present. As the digests were not carried out at the optimum pH of Z-enzyme, the number of bonds broken by this enzyme would obviously be small. In the initial stages of the attack by β-amylase and Z-enzyme, any hydrolytic action exerted by the latter would result in a change in the molecular-weight distribution without necessarily affecting the value of the mean sedimentation coefficient. At rather higher percentage conversions into maltose, the most abundant species present is still likely to be the limit dextrin, which has the same sedimentation coefficient as the original amylose. [This reasoning is based on the assumption that, at high conversions, the rate-controlling factor will be hydrolysis by Z-enzyme (cf. above).]

Conclusions.—We have shown that four different samples of β-amylase contain a second amylolytic enzyme. This enzyme is not inhibited by mercuric chloride, has no apparent effect on the β-amylolysis limit of amylopectin under normal digest conditions, and is

inactivated by digestion at pH 3·6. Further, the enzyme exerts a random hydrolytic action on amylose, a limited hydrolysis of a few bonds in amylopectin, but is unable to attack glycogen.

Thus the action pattern of Z-enzyme on amylose and amylopectin is indistinguishable from that of an α-amylase. Its apparent inability to attack glycogen, however, differentiates it from normal α-amylases.

The usual source of the enzyme is the ungerminated seed.[21] In the dormant state seeds do not contain α-amylase,[22] but there is no doubt that it appears on germination. Z-Enzyme could therefore be the dormant form of α-amylase. (It is of interest that Baba and Kojima [23] suggested that the Z-enzyme present in preparations of crude emulsin is an α-amylase.)

As Z-enzyme is non-specific in character, the nature of the barrier in amylose to the action of β-amylase remains unknown. It could be an artefact introduced during isolation or fractionation of the starch, or a natural modification of the amylose chain, or a mixture of both. The barrier may indeed vary according to the botanical source of the amylose. Elsewhere,[2] we have discussed these problems in detail and concluded that a combination of both a natural and an artificial barrier is most likely; artificial barriers can be inadvertently introduced during fractionation,[1] whilst phosphate groups [24] or branching may well occur naturally. These problems are being studied.

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The Molecular Properties of the Components of Starches*)

By C.T. GREENWOOD, Edinburgh/Scotland

Introduction

For fundamental studies of the molecular properties of the amylose and amylopectin components, granular starch must be isolated from its botanical source without any physical or chemical modification. We have found that starches can be isolated easily from plant tissues by aqueous extraction in the presence of mercuric chloride to inhibit enzymic activity. The resultant protein-contaminated starch can be purified by shaking an aqueous suspension with toluene, when denaturation of protein occurs at the toluene-water interface. This procedure provides a convenient and general method of reducing the protein to reasonable limits by purely physical methods which avoid any modification of the starch (1). (It is to be noted, however, that complete removal of protein cannot be achieved without some chemical treatment of the starch. Further, some starches are more difficult to purify than others. The significance of this is not known.)

*) This is Part XXII in the Series "Physicochemical Studies on Starches".

The ratio of the apparent amounts of the amylose and amylopectin components in such purified granular starches can vary. Continuing our studies of some of the molecular properties of the components, we have now examined this ratio for a wide variety of laboratory-prepared starches from the following sources:

Fruit and fruit-seeds.- apple, banana, and the seeds of the rubber and mango trees.

Pulses.- broad beans, smooth-seeded peas and wrinkled-seeded peas.

Cereals.- barley, oats, sweet corn, waxy maize, and wheat.

Roots and rhizomes.- iris rhizomes, parsnips and potatoes.

Many of these have not previously been examined in detail.

Table 1 shows a summary of the results. There would appear to be very large differences in iodine affinity between the starches. A starch may contain, in fact, any percentage of amylose, and there is no simple relation between this and the botanical source.

Fractionation of starches

Of the various methods of achieving fractionation (9), the most satisfactory is that involving dispersion of the granules, followed by precipitation of the amylose as a

complex with some polar organic molecule. The resultant amylose-complex may be impure, but it can be purified easily by reprecipitation. We have found (10) that thymol is useful for the first precipitation as it tends to produce purer amylopectin than other precipitants. Butan-1-ol is then very satisfactory for recrystallization. Careful fractionation in this way results in pure total amylose and amylopectin of purity $> 99\%$, but there are two essential prerequisites; the starch granules must be completely dispersed, and the presence of oxygen must be avoided.

If the granules are not completely dispersed then the separation of amylose will be poor. Certain starches are - from this point of view - difficult to handle. Perhaps the worst of these are cereal and pea starches. This behaviour may be related to their higher amylose-content. However, this difficulty can be overcome by a suitable pretreatment; the most satisfactory method of ensuring complete dispersion is to treat the granules with liquid ammonia (1). This pretreatment completely destroys crystallinity, but causes no modification in the properties of the resultant amylose component (1), and preliminary experiments suggest that the same is true for the amylo-pectin (11). The presence of oxygen during fractionation

causes both a decrease in molecular size (10) and some modification in the structure of the amylose (12).

The starches shown in Table 1 have been fractionated by methods avoiding these difficulties (1,10).

Properties of the Total Amylose Components

The various amylose samples have been characterized by measurements of (i) their apparent molecular size as shown by viscosity measurements, and (ii) their conversion into maltose under the action of purified β-amylase. The results are shown in Table 2. Although measurements of molecular size by viscosity are purely relative, some idea of the order of magnitude of the degree of polymerization ($\overline{DP}$) has been given in the Table. Values of $\overline{DP}$ have been calculated from the relation $[\eta] \times 7.4 = \overline{DP}$, which was derived from a study of potato amylose samples (10). (As the hydrodynamic behaviour of amylose may well vary from starch to starch, this relation may not be generally applicable. Experiments to investigate this are in progress.)

It can be seen that there are large differences in apparent molecular size for the various samples. Further, in no case is conversion to maltose complete under the action

of β-amylase. The presence of an apparent barrier might therefore be a characteristic of all starches, but the extent to which the barrier occurs is dependent on the botanical source. The conversion is not exactly reproducible for amyloses isolated from different samples of the same variety of starch, as it depends on the maturity of the starch (5, and see also below).

The Subfractionation of Amylose

A further complication is that amylose is heterogeneous and can be separated into fractions with varying β-amylolysis limits. The evidence for this has been recently summarized (13). It would appear that there are at least two types of amylose molecules, (i) those of relatively low $\overline{DP}$ (≈ 2000) with no structural anomaly and consequently a β-limit of 100%, and (ii) those of larger $\overline{DP}$ (up to 6000) which may well have a randomly-situated barrier as their β-limit tends to 50%. However, the distribution of molecules containing a structural anomaly in relation to the actual molecular weight distribution of the whole sample is not yet known.

Amylose in the granule can be subfractionated by aqueous leaching at varying temperatures. (It has been found that

if the fractions are to vary in their β-limit, the starch must be treated with boiling aqueous methanol (80%) (1,13).) Results obtained by this method for potato, oat, wheat and barley starches have already been given (13). Table 3 shows some comparable results for the starches from bananas, iris rhizomes and mango tree seeds. A subfraction of amylose with a β-amylolysis limit of 50% is not actually obtained, but there is a tendency towards this value. This result is not surprising in view of the obvious inefficiency of such a leaching process.

Another method which we have used recently involves a molecular weight subfractionation of the total amylose from a dispersion. This can be achieved very satisfactorily by the method of EVERETT and FOSTER (14). Amylose can be dissolved in dimethylsulphoxide and fractionally precipitated with ethanol at 4°C. In this manner, we have obtained from a sample of potato amylose (11), fractions with values of limiting viscosity number $[\eta]$ of from 80 to 1050 (c in g./ml.), and with corresponding β-amylolysis limits in the range of 100% to 60%. The fractionation conditions do not cause degradation or modification of the amylose, as the calculated average values of both $[\eta]$ and β-limit for the fractions are the same as those for the original material.

This result substantiates those from our earlier subfractionation experiments on the granules. However, in the case of the subfractions obtained by precipitation, there is not a gradual increase in the β-amylolysis limit with increase in $[\eta]$. The distribution of material containing a barrier cannot be easily deduced from such experiments, as simple molecular weight fractionation will only occur for an essentially linear series of polymer homologues. If there is any degree of branching in amylose, the factors governing precipitation will be complex. This problem is discussed in detail below.

The Nature of the Structural Anomaly in Amylose

In an earlier part of this Series, the nature of the structural anomaly, which prevents complete β-amylolysis of amylose, has been discussed (13). This barrier can be effectively removed under the concurrent action of β-amylase and Z-enzyme (15). Evidence to date shows that barriers to β-amylase action may be inadvertently introduced during fractionation (12), and natural barriers may co-exist. (The former may account for the fact that our conversion limits are some 5-10% higher than others in the literature, as precautions are taken to eliminate the presence of oxygen during fractionation.) More recently, the nature of

Z-enzyme was established for the first time (16). It is α-amylolytic in action. Although the isolation of Z-enzyme was not achieved, solutions were prepared by selectively inhibiting the β-amylase in samples containing both enzymes. (Most preparations of β-amylase do contain, in fact, Z-enzyme as a contaminant.) Inhibition was achieved with mercuric chloride.

Careful viscosity measurements showed that (i) the action of Z-enzyme on amylose containing a barrier was consistent with random hydrolysis, and (ii) amylose containing no barrier was degraded similarly. Further, lightscattering measurements showed that (i) amylopectin β-limit dextrin was degraded by the action of Z-enzyme to the extent of the scission of a few bonds per molecule, and (ii) glycogen β-limit dextrin was not attacked. These results showed that the action of Z-enzyme on amylose and amylopectin β-limit dextrin was indistinguishable from that of α-amylase. Its inability to degrade glycogen β-limit dextrin distinguishes it from normal α-amylase.

It is of particular interest that as the action of the enzyme is limited to the hydrolysis of only a few bonds per molecule, chemical methods of investigation such as iodine-stain and reducing power estimations are unsuitable. This is particularly true for the latter measurements,

especially when Somogyi-type reagents are used. (See also comments in ref. 1.)

It should be noted perhaps that for amylose containing a barrier, the observed β-amylolysis limit when β-amylase follows Z-enzyme will not necessarily be 100%. The effect of the concurrent action of β-amylase and Z-enzyme is necessarily different from that of β-amylase following Z-enzyme. If amylose is considered as a mixture of linear molecules and molecules containing a random-barrier, when both enzymes are present the high turn-over number of the β-amylase (17) will ensure that all available substrate is removed i.e. linear molecules will be completely degraded and those with a barrier will be degraded to ca. 50%. Further β-amylolysis will then occur as the resistant portions are concurrently hydrolysed by Z-enzyme. Thus the concurrent action of both enzymes will result rapidly in 100% conversion. When Z-enzyme acts alone, however, it will hydrolyse a random selection of the amylose molecules. Hydrolysis of a linear molecule will cause no change in the observed limit on subsequent β-amylolysis, but hydrolysis of a molecule containing a barrier will necessarily increase the β-limit. The final conversion will be dependent simply on the extent of hydrolysis, which will be governed itself by the concentration of Z-enzyme and the pH of the digest (cf. ref. 16).

The discovery that Z-enzyme is non-specific makes the problem of the nature of the barrier more difficult. It might well vary from starch to starch. Some form of natural barrier appears very probable. We have shown that the extent of β-amylolysis for potato amylose depends on the maturity of the starch (5). A more recent and extensive study of the properties of the components during the growth of starch granules in the pea plant (2) has shown that the apparent size of the amylose increases whilst its extent of β-amylolysis decreases (see Table 4). The observed extent of β-amylolysis of any amylose is therefore strongly dependent on the maturity of the starch granule.

The most likely possibilities for natural barriers are phosphate ester groupings and/or very limited branching. Our preliminary experiments with phosphatase (11) have indicated, however, that in agreement with PEAT, THOMAS and WHELAN (15), phosphate ester groupings are not responsible for the barrier. One approach to the problem of whether there is branching in amylose is to study the hydrodynamic behaviour of amylose subfractions of varying molecular weight and β-amylolysis limit.

The Hydrodynamic Behaviour of Amylose

Very few studies have been made of the hydrodynamic behaviour of amylose - either for the free component or for derivatives - and the results obtained have not been in agreement (see ref. 9). Recently some extensive work on subfractions of potato amylose has been carried out by EVERETT and FOSTER (18). By means of their new subfractionation technique (14) mentioned above, various samples were obtained and examined by viscosity and lightscattering. The results, which enabled the exponent in the modified Staudinger equation to be determined, suggested that amylose behaved as a random coil in the solvents studied. However, these authors made no attempt to characterize the fractions by their β-amylolysis limits.

We have subfractionated potato amylose by this method (11); some 10 fractions were obtained and characterized by measurements of the β-limit, $[\eta]$, and weight-average molecular weight ($\bar{M}_w$). The latter values were obtained from lightscattering measurements in aqueous potassium chloride. Lightscattering data were obtained by ZIMM's treatment (19), and we have confirmed that in this solvent, the second virial coefficient is negligible (14). Values of $\bar{M}_w$ for our fractions ranged from 160,000 to 14,400,000. As indicated above, there is no direct relation between β-limit and $[\eta]$ for these

fractions. The graph of log $[\eta]$ versus log $\bar{M}_w$ for the linear fractions has a slope of 0.56 (see Fig. 1). This confirms EVERETT and FOSTER's results (18) that the polymer is behaving as a random coil. But it is of interest that the points for all the modified fractions lie significantly below the curve i.e. $\bar{M}_w$ is very much greater than expected from the $[\eta]$-value. As all the fractions are >98% pure, this result suggests that limiting long-chain branching may be occurring in the modified fractions. Furthermore, this behaviour was confirmed by sedimentation measurements.

The hydrodynamic behaviour of amylose fractions would suggest therefore that (i) linear amylose molecules behave as random coils in aqueous salt solution, and (ii) amylose molecules with a barrier to β-amylolysis may well contain branches. Experiments to establish the distribution of molecules containing a barrier over the molecular weight distribution for the whole sample are in progress.

Properties of the Amylopectin Components

The amylopectin components isolated from aqueous dispersions of many of the starches shown in Table 1 have been characterized by measurements of their purity, average length of unit-chain, β-amylolysis limit and phosphorus-content. These results are shown in Table 5. In all cases,

the amount of amylose impurity was small (< 2% as shown by measurements of iodine affinity). The average lengths of unit-chain obtained by periodate oxidation show significant differences between the various samples. The value would appear to depend on the source, as it is reproducible for different samples of the same amylopectin. However, the β-amylolysis limits for the different samples are not significantly different. There are large differences in the phosphorus-content of the samples. This influences some of the physical properties of amylopectin solutions as discussed below.

Consistently high molecular weights have been obtained for amylopectin by many workers (9), and the question inevitably arises of whether these large values are real or the result of aggregation. Most determinations of $\bar{M}_w$ have been made by lightscattering and, therefore, in addition to the possibility of aggregation, it is possible that small quantities of undispersed starch granules might influence the results. However, both these possibilities appear to have been ruled out by the work of WITNAUER, SENTI and STERN (20) on potato amylopectin, of STACY and FOSTER (21) on maize amylopectin, and of ERLANDER and FRENCH (22) on maize and waxy maize amylopectin.

We have carried out investigations on potato amylopectin. This material was chosen primarily as it can be obtained in a very pure state (<0.5% of amylose-contaminant), but also as earlier work (5) had shown it to have unique properties in that the slope of the graph of the inverse of the sedimentation coefficient versus the concentration was not reproducible. Potato amylopectin contains more ester-phosphate groupings (see SAMEC (23)) than is usual, and hence these ionizable groups must confer some polyelectrolyte character. The extent of this has been investigated by measuring viscosity, sedimentation and lightscattering of amylopectin in solutions of sodium chloride of concentration 1M to 10^{-5}M (24).

A carefully dialysed sample of potato amylopectin was used. Sedimentation results showed a very large dependence of sedimentation coefficient on concentration, but the dependence was considerably decreased in salt solutions. An interesting consequence of this was the corresponding increased spreading of the sedimentation boundary. In water solution, very sharp boundaries were obtained due to the strong JOHNSTON-OGSTON (25) and boundary sharpening effects. In salt solution these were much reduced, and the shape of the boundary approached more closely to that expected for a polymer of very wide molecular weight distribution.

The viscosity of amylopectin solutions was profoundly affected by salt concentration. $[\eta]$ in 10^{-5}M sodium chloride was effectively the same as in pure water, but in 10^{-2}M salt it had fallen to about 1/4 of this value. At the same time, the concentration dependence of η_{sp}/C fell considerably and in the stronger salt solutions was effectively independent of concentration.

Lightscattering measurements showed that in the concentration range examined (1-20 x 10^{-5} g./ml.), the second virial coefficient was negligible. Data were again treated as suggested by ZIMM (19). This enabled both $\bar{M}_w$ and the radius of gyration to be evaluated. The sample of potato amylopectin studied was found to have a molecular weight of ca. 500 x 10^6. This value was reproducible and independent of the solvent. (It was dependent, however, on the force-field used to clarify the solution.) The observed radius of gyration depended on the solvent; in water the value was 6400Å, whilst in 0.1M sodium chloride it was 3800Å.

The volume of the molecule is thus considerably less in salt solutions. This is further reflected in the corresponding reduction of concentration dependence of both viscosity and sedimentation coefficient. These effects must be due to the presence of ester phosphate groups in the amylopectin. A possible explanation of these phenomena is based on the mutual

repulsion of the negatively charged phosphate groups. In pure water, the repulsive effect will be largely unhindered and the molecule is expanded. The addition of electrolyte surrounds each phosphate group with an atmosphere of cations and the consequent screening suppresses the repulsion and allows the molecule to assume a more contracted form. The extent to which this behaviour occurs is obviously dependent on the amount of phosphate in the amylopectin sample. This has been confirmed by a study of other samples of amylopectin.

Very few previous results for the molecular weight of potato amylopectin have appeared. WITNAUER, SENTI and STERN (20) found 36×10^6. However, the parent starch was a commercial sample and was dispersed in air. Degradation was therefore inevitable, and it is thought that the value of 500×10^6 is more representative of native amylopectin. Amylopectin must therefore be one of the largest molecules found in Nature.

Summary

Starches from a wide variety of botanical sources have been isolated, purified and fractionated into their amylose and amylopectin components. Some molecular properties of the fractions have been examined.

In the case of the pure amyloses, in addition to apparent variations in molecular size, as shown by differences in viscosity, there are large differences in the extent of β-amylolysis. These differences appear to be a characteristic of the source of starch. All amyloses are heterogeneous and can be separated into fractions with varying β-amylolysis limits. Recent experiments to identify the nature of the barrier to β-amylase are described. The limitations of enzymic methods to solve this problem are emphasised, and the results of recent physicochemical techniques are discussed.

The amylopectin components have been characterized by measurements of their purity, average length of unit-chain, β-amylolysis limit and phosphorus-content. Further, viscosity and lightscattering measurements have been made in a variety of solvents. The latter measurements enabled calculations of molecular weights and radii of gyration to be made. The solution behaviour of amylopectins is strongly influenced by the presence of phosphate groups. Examples of this behaviour are given and discussed.

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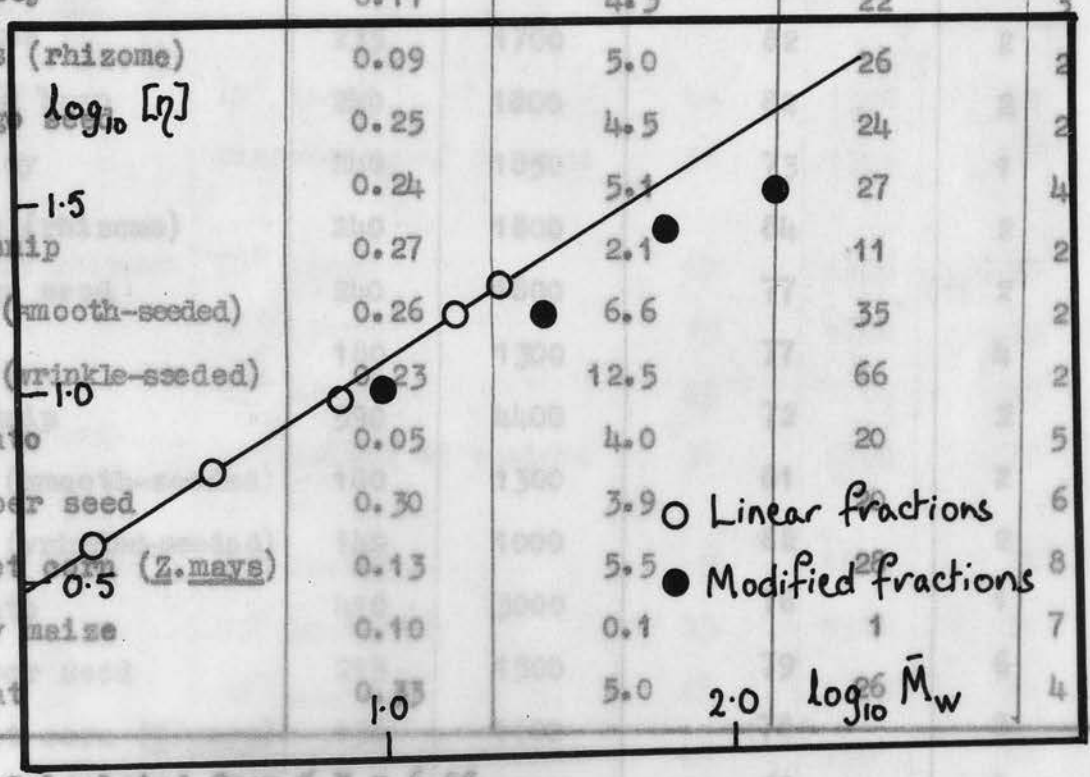
Table 1

The Iodine Affinity and Apparent Amylose Content of Some Starches

Starch	Protein (%) [1]	Iodine affinity (%) [2]	Apparent Amylose-Content (%) [3]	Ref.
Apple	0.10	3.6	19	2
Banana	0.32	5.0	18	2
Broad bean	0.16	4.5	24	2
Barley	0.11	4.3	22	3
Iris (rhizome)	0.09	5.0	26	2
Mango	0.25	4.5	24	2
Oat	0.24	5.1	27	4
Parsnip	0.27	2.1	11	2
Pea (smooth-seeded)	0.26	6.6	35	2
Pea (wrinkle-seeded)	0.23	12.5	66	2
Potato	0.05	4.0	20	5
Rubber seed	0.30	3.9	20	6
Sweet corn (Z. mays)	0.13	5.5	28	8
Waxy maize	0.10	0.1	1	7
Wheat	0.33	5.0	26	4

FIG. 1. Graph of $\log_{10}[\eta]$ versus $\log_{10}\bar{M}_w$ for amylose fractions.



1) Calculated from % N x 6.25.

2) Expressed as mg. I_2 bound/100 mg. starch; $[I^-] = 10^{-2}$M; $[\text{total free iodine}]_{max} = 1 \times 10^{-5}$M; pH = 5.85.

3) Calculated assuming that the iodine affinity of pure amylose = 19.0%, except for potato amylose where a value of 19.5 was used (10).

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Mango seed	0.25	4.5	24	2
Oat	0.24	5.1	27	4
Parsnip	0.27	2.1	11	2
Pea (smooth-seeded)	0.26	6.6	35	2
Pea (wrinkle-seeded)	0.23	12.5	66	2
Potato	0.05	4.0	20	5
Rubber seed	0.30	3.9	20	6
Sweet corn (Z. mays)	0.13	5.5	28	8
Waxy maize	0.10	0.1	1	7
Wheat	0.33	5.0	26	4

1) Calculated from % N x 6.25.

2) Expressed as mg. I_2 bound/100 mg. starch; $[I^-] = 10^{-2}$M; $[\text{total free iodine}]_{max} = 1 \times 10^{-5}$M; pH = 5.85.

3) Calculated assuming that the iodine affinity of pure amylose = 19.0%, except for potato amylose where a value of 19.5 was used (10)

Table 2

The Properties of Amylose Fractions obtained from Dispersions of the Granules of Some Starches

Starch	$[\eta]$ in MKOH	Apparent $\overline{DP}$ [1]	% conversion to maltose on β-amylolysis	Ref.
Apple	200	1500	84	2
Banana	235	1700	82	2
Broad bean	240	1800	82	2
Barley	248	1850	73	1
Iris (rhizome)	240	1800	84	2
Mango seed	240	1800	77	2
Oat	180	1300	77	4
Parsnip	590	4400	72	2
Pea (smooth-seeded)	180	1300	81	2
Pea (wrinkled-seeded)	140	1000	82	2
Potato	410	3000	76	1
Rubber seed	215	1500	79	6
Sweet corn (Z. mays)	150	1100	78	8
Wheat	280	2100	68	1

1) Calculated from $\overline{DP} = 7.4 \times [\eta]$, where $[\eta]$ is in ~~dl/g~~ g./ml.

Table 3

Properties of Amylose Subfractions obtained by Successive Leaching of Granules treated with 80% Aqueous Methanol (2)

Starch	Procedure	% of amylose extracted	D.P.	β-limit
Banana	70° leach	44	1300	99
	Dispersion of residue	56	1900	62
Iris rhizome	70° leach	19	1400	98
	80° leach	25	1700	89
	90° leach	25	1900	76
	Dispersion of residue	31	2100	72
Mango tree seeds	70° leach	9	1400	98
	80° leach	25	1500	89
	90° leach	25	1700	72
	Dispersion of residue	41	1900	68

Table 4.

The Properties of Amyloses obtained from Dispersions of Pea Starches of varying Maturity (2)

Growth stage	1	2	3	4
$[\eta]$ in 1MKOH	145	160	195	210
β-amylolysis limit	90	86	79	74

Table 5.

The Properties of Amylopectin Fractions obtained from Dispersions of the Granules of Some Starches.

Starch	% of Phosphorus	Average length of unit-chain	β-Amylolysis limit	Ref.
Apple	0.025	22	58	2
Banana	-	21	59	2
Broad bean	0.017	23	57	2
Barley	-	25	58	2
Iris rhizome	0.048	22	57	2
Mango tree seed	0.012	21	56	2
Parsnip	0.054	21	58	2
Pea (smooth-seeded)	-	26	58	2
Pea (wrinkled-seeded)	-	27	58	2
Potato	0.111	24	56	5
Rubber tree seed	-	23	-	6
Sweet corn (Z. mays)	-	23	-	8